

Cell Therapy for Temporal Lobe Epilepsy:

Feasibility of Neurotransplantation

versus

Modulation of Endogenous Neurogenesis

Robrecht Raedt

Thesis submitted to fulfil the requirements for the degree of Doctor in Medical Sciences
August 2007



Laboratory for Clinical and
Experimental Neurophysiology



Onderzoek gefinancierd met een specialisatiebeurs van het instituut voor de aanmoediging van Innovatie door Wetenschap en Technolgie in Vlaanderen (IWT-Vlaanderen, beurs nummer 23363)

Research funded by a PhD grant of the Institute for the promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen, grant number 23363)

Promotor

Prof. Dr. Paul Boon

Universiteit Gent, Belgium

Copromotor

Prof. Dr. J. Plum

Universiteit Gent, Belgium

Members of the examination committee

Prof. Dr. Marleen Temmerman

Universiteit Gent, Belgium

Prof. Dr. Robert Rubens

Universiteit Gent, Belgium

Prof. Dr. Catherine Verfaillie

Katholieke Univeriteit Leuven, Belgium

Prof. Dr. Elinor Ben-Menachem

Goteborg University, Sweden

Prof. Dr. Wytse Wadman

Universiteit van Amsterdam, The Netherlands

Prof. Dr. Bart Vandekerckhove

Universiteit Gent, Belgium

Prof. Dr. Kristl Vonck

Universiteit Gent, Belgium

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION ON TEMPORAL LOBE EPILEPSY	9
EPILEPSY	11
TEMPORAL LOBE EPILEPSY	12
ANIMAL MODELS FOR EPILEPSY	16
CHAPTER 2: CELL THERAPY IN NEUROLOGY	27
MANUSCRIPT 1: <u>RAEDT R. & BOON P.</u> , CELL THERAPY FOR NEUROLOGICAL DISORDERS: A COMPREHENSIVE REVIEW29 <i>ACTA NEUROL BELG. 2005,105,158-170</i>	
ADDENDUM TO MANUSCRIPT 1	51
MANUSCRIPT 2: <u>RAEDT R. ET AL.</u> , CELL THERAPY IN MODELS FOR TEMPORAL LOBE EPILEPSY71 <i>SEIZURE. JUNE 11 2007, ONLINE EARLY ARTICLE</i>	
CHAPTER 3: ADULT NEUROGENESIS	97
INTRODUCTION ON ADULT NEUROGENESIS	99
ADULT NEUROGENESIS AND NEURAL DAMAGE	103
NEUROGENESIS IN EPILEPSY	107
CHAPTER 4: RATIONALE, AIMS AND OUTLINE OF THE THESIS	115
RATIONALE	117
AIMS	121
OUTLINE OF THE THESIS	123
CHAPTER 5: CELL TRANSPLANTATION FOR TLE	127
MANUSCRIPT 3: <u>RAEDT R. ET AL.</u> , DIFFERENTIATION ASSAYS OF BONE MARROW DERIVED MAPC-LIKE CELLS129 TOWARDS NEURAL CELLS CANNOT DEPEND ON MORPHOLOGY AND A LIMITED SET OF NEURAL MARKERS <i>EXPERIMENTAL NEUROLOGY. 2007,203,542-554</i>	
MANUSCRIPT 4: <u>RAEDT R. ET AL.</u> , SEIZURES IN THE INTRAHIPPOCAMPAL KAINIC ACID EPILEPSY MODEL:.....155 CHARACTERIZATION USING LONG TERM VIDEO-EEG MONITORING IN THE RAT <i>ACTA NEUROLOGICA SCANDINAVICA. 2007,SUBMITTED</i>	
MANUSCRIPT 5: <u>RAEDT R. ET AL.</u> , TRANSPLANTATION OF ADULT SUBVENTRICULAR ZONE-DERIVED NEURAL175 STEM CELLS IN THE KAINIC ACID LESIONED HIPPOCAMPUS OF THE RAT <i>EXPERIMENTAL NEUROLOGY. 2007,SUBMITTED</i>	
CHAPTER 6: MODULATION OF ENDOGENOUS NEUROGENESIS IN TLE	199
MANUSCRIPT 6: <u>RAEDT R. ET AL.</u> , RADIATION OF THE RAT BRAIN SUPPRESSES SEIZURE-INDUCED NEUROGENESIS.....201 AND TRANSIENTLY ENHANCES EXCITABILITY DURING KINDLING ACQUISITION <i>EPILEPSIA. JUNE 6 2007,ONLINE EARLY ARTICLE</i>	
CHAPTER 7: GENERAL DISCUSSION	229
CELL THERAPY FOR TLE THROUGH CELL TRANSPLANTATION	231
CELL THERAPY FOR TLE THROUGH MODULATION OF ENDOGENOUS NEUROGENESIS	249
CHAPTER 8: GENERAL CONCLUSION	261

CHAPTER 9: FUTURE PERSPECTIVES	271
CELL TRANSPLANTATION FOR TLE.....	273
MODULATION OF ENDOGENOUS NEUROGENESIS IN CASE OF TLE	276
SUMMARY; SAMENVATTING; RÉSUMÉ	283
DANKWOORD	295

LIST OF ABBREVIATIONS

4-VO	four vessel occlusion	DCX	doublecortin
6-OHDA	6-hydroxydopamine	DMEM	dulbecco's modified eagle's medium
ABI	applied biosystems	DMSO	dimethylsulfoxide
AchE	acetylcholine	DNA	deoxyribonucleic acid
AD	afterdischarge	DPBS	dulbecco's phosphate buffered saline
ADD	afterdischarge duration	DV	dorsoventral
ADK	adenosine kinase	EC	embryonic carcinoma
ADT	afterdischarge threshold	EEG	electro-encephalogram
AED	antiepileptic drugs	EG	embryonic germ
ALS	amyotrophic lateral sclerosis	EGF	epidermal growth factor
ang	angiopoietin	EPSP	excitatory postsynaptic potentials
ANOVA	analysis of variance	ESC	embryonic stem cells
AP	anteroposterior	ESGP	embryonic stem cell-derived glial precursors
araC	cytosine-b-D-arabinofuranoside	ESN	embryonic stem cell-derived neurons
azaC	5-azacytidine	FBS	fetal bovine serum
β -3 tub	β -3 tubulin	FCS	fetal calf serum
BDNF	brain-derived neurotrophic factor	FF	fimbria fornix
bFGF	basic fibroblast growth factor	FGF	fibroblast growth factor
BHA	butylated hydroxyanisol	FSG	fish skin gelatin
BLA	basolateral amygdala	GABA	gamma-aminobutyric acid
BLAST	basic local alignment search tool	GAD	glutamic acid decarboxylase
BM	bone marrow	GalC	galactocerebrosidase
BMMNC	bone marrow mononucleated cells	GCL	granule cell layer
BMP	bone morphogenic protein	GDNF	glial-derived neurotrophic factor
BMSC	bone marrow-derived multipotent cells	GFAP	glial fibrillary acidic protein
BrdU	5-bromo-2 deoxyuridine	GFP	green fluorescent protein
CA	cornu ammonis	GKS	gamma knife surgery
cDNA	copy deoxyribonucleic acid	Gy	gray
CFW	california fine wire	HD	Huntington's disease
CNS	central nervous system	HFO	high frequency oscillation
CNTF	ciliary neurotrophic factor	HPLC	high pressure liquid chromatography
CPS	complex partial seizures	HPRT	hypoxanthine phosphoribosyl transferase
DAPI	4,6-diamidino-2-phenylindole	HS	hippocampal sclerosis
db-cAMP	dibutyl cyclic adenosine monophosphate	HSC	hematopoietic stem cells
DBS	deep brain stimulation	HUVEC	human umbilical vein endothelial cells

HYP	hypersynchronous type	NT-3	neurotrophin-3
ICC	immunocytochemistry	PB	phosphate buffer
IGF	insulin growth factor	PBS	phosphate buffered saline
IgG	immunoglobulin	PC	pyriform cortex
ILAE	international league against epilepsy	PD	Parkinson's disease
IR	immunoreactive	PDGF	platelet-derived growth factor
ITS	insulin-transferrin-selenium	PFA	paraformaldehyde
KA	kainic acid	PGC	primordial germ cells
LA-BSA	linoleic acid-bovine serum albumin	PILO	pilocarpine
LC	locus coeruleus	PLD	postlesion delay
LTLE	lateral temporal lobe epilepsy	PPV	posterior periventricular region
LVF	low-voltage fast type	PSA-NCAM	polysialylated neural cell adhesion molecule
MACS	magnetic beads cell sorting	PTZ	pentylene-tetrazol
MAP	microtubule associated protein	RA	retinoic acid
MAPC	multipotent adult progenitor cells	RFA	radiofrequency ablation
MBP	myelin basic protein	RMS	rostral migratory stream
MES	maximal electroshock	RNA	ribonucleic acid
MHC	major histocompatibility complex	RT-PCR	real-time polymerase chain reaction
MIAMI	marrow-isolated adult multilineage inducible	SCG	superior cervical ganglion
ML	mediolateral	SE	status epilepticus
mLIF	mouse leukemia inhibitory factor	SEM	standard error of the mean
MOSFET	Metal-Oxide-Semiconductor Field-Effect Transistor	SGZ	subgranular zone
MRI	magnetic resonance imaging	SG	secondary generalization
mRNA	messenger ribonucleic acid	SHH	sonic hedgehog
MS	medial septum	SNr	substantia nigra
MSC	mesenchymal stem cells	SPS	simple partial seizures
MTLE	mesial temporal lobe epilepsy	SSSE	self-sustaining status epilepticus
NA	noradrenaline	SVZ	subventricular zone
NCBI	national centre for biotechnology information	TBS	tris-buffered saline
NeuN	neuronal nuclei	TH	tyrosine hydrolase
NF	neurofilament	TLE	temporal lobe epilepsy
NGF	nerve growth factor	TX100	Triton X-100
NMDA	N-methyl-D-aspartate	USSCs	unrestricted somatic stem cells
NSC	neural stem cells	VEGF	vascular endothelial growth factor
NSE	neuron specific enolase	VNS	vagus nerve stimulation

Chapter 1

Introduction on Temporal Lobe Epilepsy

EPILEPSY

Epilepsy affects 0.5-1% of the general population and is the second most common chronic neurological disease following cerebrovascular disorders. It is a central nervous system (CNS) disorder characterized by recurring epileptic seizures and by the neurobiological, cognitive and psycho-social consequences for individual patients. The definition of epilepsy requires the occurrence of at least two epileptic seizures.

An epileptic seizure is defined as a transient occurrence of signs and symptoms due to excessive or abnormal synchronous neuronal activity in the brain (Fisher and others, 2005). The classification of epileptic seizures is based on clinical symptoms and electrical brain activity as proposed by the Commission of Classification and Terminology of the International League Against Epilepsy (ILAE) (Anonymous, 1981; Anonymous, 1985; Anonymous, 1989). Seizures can be classified as partial, when they are limited to a part of one cerebral hemisphere, or generalized, when both hemispheres are involved in seizure induction.

Partial seizures are the most common form of epileptic seizures and can be subdivided on the basis of whether or not consciousness is impaired during the attack and whether or not progression to generalized convulsions occurs. Simple partial seizures (SPS) are seizures in which consciousness is not impaired. In complex partial seizures (CPS) consciousness is impaired. A partial seizure that does not terminate but instead progress to a generalized motor seizure is termed a partial seizure with secondary generalization (SG).

Generalized seizures can be subdivided into absence seizures, myoclonic seizures, clonic seizures, tonic seizures, tonic-clonic seizures and atonic seizures.

Epilepsy syndromes can also be classified according to the underlying etiology as idiopathic when they are not associated with a brain lesion or symptomatic when they result from a known or suspected disorder of the brain (Panayiotopoulos, 2005).

TREATMENT OF EPILEPSY

Chronic administration of antiepileptic drugs (AED) is the treatment of choice for epilepsy. Kwan and Brodie have shown that the first AED leads to seizure freedom in 47% of patients with newly diagnosed epilepsy. Thirteen percent are seizure free with the second AED, and only 1% after switching to a third AED (Kwan and Brodie, 2001). In general, about 30% to 40% of all epilepsy patients have uncontrolled seizures or unacceptable medication-related side effects despite adequate pharmacological treatment. These patients have 'refractory epilepsy' (Brodie and Kwan, 2002). It is however very important to provide seizure control in order to prevent cognitive deterioration (Boon and others, 1994; Jokeit and Ebner, 2002), ongoing neuronal damage (Sutula and others, 2003) or sudden unexpected death (Tomson and others, 2005). Therefore research towards alternative therapies to treat refractory seizures is ongoing.

The least invasive treatment option consists of including patients in trials with *newly developed antiepileptic drugs*. These result into seizure freedom in about 7% of patients with refractory epilepsy (Fisher, 1993).

Epilepsy surgery is an invasive but often curative treatment option that aims at removing the ictal onset zone believed to be responsible for seizure occurrence (Wiebe and others, 2001). Depending on the localization of the seizure focus, approximately 70% of the patients are rendered seizure free after resective epilepsy surgery (Engel, 1996a). However, a large number of patients, included for presurgical evaluation, are unsuitable surgery candidates due to the absence of a circumscribed ictal onset zone or the localisation of the onset zone in functional brain tissue (Boon and others, 1999; Clusmann and others, 2002).

Gamma knife surgery (GKS) and *radiofrequency ablation (RFA)* are less invasive alternatives for resective surgery. GKS is based on the convergence of 201 gamma ray beams. Cortical structures can be targeted with GKS with a stereotactic precision, without opening the skull. RFA requires putting an electrode in the structure that needs to be lesioned, but is still less invasive than complete resection. Until now, no definite conclusions can be made concerning efficacy and safety of both approaches (Parrent and Blume, 1999; Regis and others, 2004; Srikijvilakul and others, 2004).

The *ketogenic diet* consists of a high-fat, adequate protein and low-carbohydrate diet that mimics the biochemical changes associated with starvation, which creates ketosis. This treatment is sometimes associated with severe systemic side effects. Long-term prospective studies show a reduction of seizure frequency of more than 90% in one third of the patients who are able to continue the diet (Freeman and others, 1998; Freeman and others, 2006).

Vagus nerve stimulation (VNS) has proven to be an efficacious and safe treatment during long-term follow-up (Vonck and others, 2004). Through an implanted device and an electrode wound around the left nervus vagus in the neck, electrical pulses are administered to the afferent nerve fibers. VNS is indicated in patients with refractory epilepsy who are unsuitable candidates for epilepsy surgery or who have had insufficient benefit from such a treatment (Ben-Menachem, 2002).

Another method of neurostimulation involves direct stimulation of deep brain structures, termed *deep brain stimulation (DBS)*. DBS requires insertion of stimulation electrodes into intracerebral targets. Although our group and others have demonstrated efficacy and safety of chronic DBS as an alternative treatment for refractory temporal lobe epilepsy (TLE) (Vonck and others, 2002; Velasco and others, 2005), this technique is still in an early stage of development.

TEMPORAL LOBE EPILEPSY

Temporal lobe epilepsy (TLE) comprises 30-35% of all epilepsies and is the most common form of refractory symptomatic epilepsy (Spencer, 2002). Patients suffering from TLE can have SPS, in which the patients remain conscious. The SPS can be divided into different subgroups: SPS with changes in muscle activity (simple partial motor seizures), changes in sensation (simple sensory seizures), autonomic changes and psychic changes. More

common are CPS in which the patients lose consciousness and have sensory, mental, visceral and somatomotor symptoms. A CPS is often preceded by an aura. Although temporal lobe seizures are always partial, they can become secondarily generalized and result into tonic-clonic seizures (Mathern and others, 1996; Engel, 1996b).

TLE can be categorized in two groups: those with seizures originating in the mesial temporal lobe structures (mesial temporal lobe epilepsy, MTLE) and those with seizures beginning elsewhere in the temporal lobe [e.g., lateral temporal lobe epilepsy (LTLE)]. There is very little information about LTLE and distinguishing seizure characteristics do not exist (Walczak, 1995). Patients suffering from LTLE comprise less than 10% of patients with TLE. Unlike patients with MTLE, there are no reported large series of patients with well-documented lateral temporal lobe seizure origin. This partly explains why LTLE is not well described (Williamson and others, 1999). Therefore, in this dissertation, we refer to MTLE when we describe TLE, unless otherwise stated.

The hypothesis on TLE development covers three phases; (1) an initial precipitating insult of the brain such as head trauma, status epilepticus (SE), stroke, inflammation or febrile seizures initiates a cascade of events; (2) a period of epileptogenesis during which several processes (either molecular or structural) occur; (3) chronic epilepsy characterized by the occurrence of recurrent seizures.

There is still a lot of debate about the mechanisms underlying development and intractability of TLE, but it is generally believed that limbic structures such as the hippocampus, the amygdala and the temporal neocortex play crucial roles. One of the reasons for this belief is the fact that surgical removal of these temporal lobe structures eliminates seizures in up to 80% of the cases (Lieb and others, 1987; Engel, Jr., 1992; Foldvary and others, 2000). Histological studies on tissue, obtained surgically from patients with intractable and unilateral TLE, reveal specific changes which could underlie hyperexcitability of the removed structures. Typical alterations that are described in temporal lobe regions involve neuronal loss (Lewis, 2005), gliosis, synaptic plasticity (Cavazos and Cross, 2006), neurogenesis (Parent, 2002), inflammation (Vezzani and others, 2002), and molecular reorganization in cellular membranes and extracellular matrix (Perosa and others, 2002; Avanzini and Franceschetti, 2003).

Hippocampal sclerosis (HS) is the most common lesion observed in patients with refractory TLE and is found in 60-70% of the cases referred for surgical evaluation (Lewis, 2005). HS is frequently the result of a previous status epilepticus, complicated febrile convulsions, encephalitis or an ischemic insult (French and others, 1993). However also the seizures themselves can cause or aggravate HS (Sutula and others, 2003). The hallmark of HS is extensive gliosis combined with a rather selective loss of neurons in the hilus of the dentate gyrus and areas CA1 and CA3 of the hippocampus proper (fig. 1). Granule cells of the dentate gyrus and pyramidal neurons of the area CA2 and the subiculum are relatively spared. Neuronal cell loss involves both glutamatergic excitatory neurons and GABAergic inhibitory interneurons in the dentate gyrus and the hippocampus proper. Sclerosis can extend to other mesiotemporal regions such as the amygdala, the entorhinal, perirhinal and temporopolar cortex (Yilmazer-Hanke and others, 2000; Jutila and others, 2001; Salmenpera and others, 2001; Hermann and others, 2002; Wieser, 2004).

There are several indications that hippocampal sclerosis plays an important contributing role in TLE. Firstly, from epilepsy surgery it is evident that best seizure control (up to 80%) in TLE is obtained when a large part of the hippocampus is included in the temporal lobe resective surgery (Mathern and others, 1995b; Engel, 1996a). Secondly, careful pathologic analysis of temporal lobe specimens, especially from patients with seizure control following resection, have found that most contain an area of focal pathology, usually hippocampal sclerosis or mass lesions (French and others, 1993). By contrast, in most surgical patients with poor seizure control, no hippocampal pathology or only mild damage can be demonstrated (e.g. amygdalar sclerosis) (Miller and others, 1994; Mathern and others, 1995b). Thirdly, intracranial EEG seizure onsets correlate with the presence of hippocampal sclerosis. Severe neuron loss in the anterior hippocampus was associated with anterior focal EEG ictal onsets. Cell death in both the anterior and posterior hippocampal segments was associated with regional mesial temporal onsets (Babb and others, 1984).

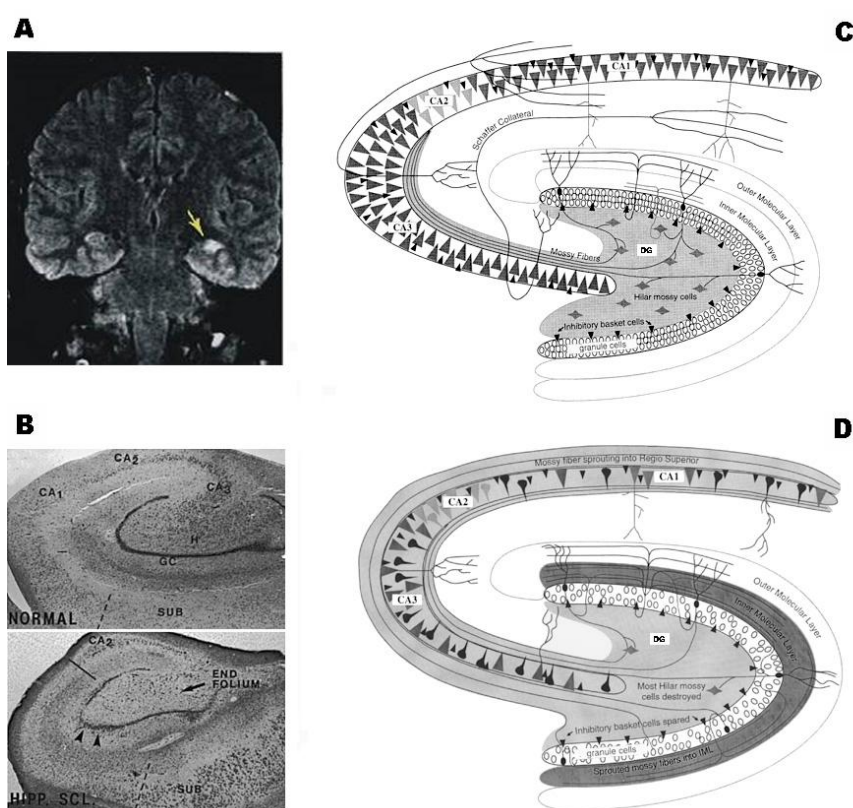


Fig 1 Hippocampal sclerosis in temporal lobe epilepsy. A) A coronal FLAIR (fluid attenuated inversion recovery) T2-weighted magnetic resonance imaging (MRI) image showing left hippocampal sclerosis (arrow). B) Nissl staining of normal (above) and sclerotic hippocampus (below). In the normal hippocampus different hippocampal subfields can be discriminated: the granule cell layer (GC) and hilus (H) of the dentate gyrus (DG); the CA1, CA2 and CA3 subfield of the hippocampus proper and the subiculum (SUB). In the sclerotic hippocampus there is extensive loss of neurons in the hilus, CA3 and CA1 regions. In the CA2 region and the subiculum cells are relatively spared. Granule cells also show damage and dispersion (arrowheads). C) Schematic illustration of normal hippocampal anatomy (above). Granule cell dendrites are located in the inner and outer molecular layers of the DG where they receive input of the entorhinal cortex through the perforant path. Activation of the granule cell axons, the mossy fibers (light-shaded gray area), can stimulate CA3 pyramidal neurons. In addition hilar mossy cells (rhomboid shaped) and inhibitory basket cells (solid triangles) are stimulated, thereby regulating granule cells by feedback mechanisms. The axons of the mossy cells are in the inner molecular layer. Axons of the CA3 neurons, the Schaffer collaterals, stimulate CA1 pyramidal neurons. Axons of CA1 neurons leave the hippocampus through the subiculum. D) In hippocampal sclerosis there is loss of hilar mossy cells, CA3 and CA1 neurons and other hippocampal cells. Mossy fibers sprout axon collaterals into the inner molecular layer (dark gray shade) and into regio superior (light shaded area). Figure modified from (Mathern and others, 1999)

Another frequently described phenomenon in both human and experimental TLE is aberrant sprouting of granule cell axon collaterals in regions they normally does not innervate (fig. 1). This process is called mossy fiber sprouting and is most probably the result of the loss of appropriate targets of the mossy fibers, e.g. hilar polymorphic neurons and CA3 neurons, during the epileptogenic process (Cavazos and Cross, 2006). The most prominent mossy fiber sprouting is seen in the inner molecular layer of the dentate gyrus (Sutula and others, 1989; Babb and others, 1991; Isokawa and others, 1993). The aberrantly sprouted mossy fibers innervate preferentially glutamatergic granule cells, creating primarily a recurrent excitatory feedback circuitry. Therefore, mossy fiber sprouting is believed to be an excitatory phenomenon (Wuarin and Dudek, 1996; Buckmaster and others, 2002; Scharfman and others, 2003). Electrophysiological studies on human hippocampal slices and experimental studies in animal models for TLE confirmed this by showing that the extent of mossy fiber sprouting correlates with the excitability of the hippocampus (Cavazos and others, 1991; Franck and others, 1995). Next to the inner molecular layer mossy fiber sprouting was also reported in the CA3 and CA1 regions of the hippocampus (Mathern and others, 1999; Cavazos and Cross, 2006).

The degree of mossy fiber sprouting correlates with the degree of neuronal loss (Cavazos and Cross, 2006) and both pathological factors seem to contribute to the intensification of chronic seizures (Gorter and others, 2001; Zhang and others, 2002). Although HS and mossy fiber sprouting are very often associated with TLE, there are patient studies (Spencer and Spencer, 1994) and experimental studies (Longo and Mello, 1997; Longo and Mello, 1998; Zhang and others, 2002; Romcy-Pereira and Garcia-Cairasco, 2003; Brandt and others, 2004) showing that temporal lobe epileptogenesis can also occur without both processes taking place. So the role of both processes in the genesis of MTLE remains controversial.

The dendritic tree of the granule cells of the dentate gyrus also exhibits morphological plasticity. In both human TLE patients (von Campe and others, 1997) and experimental animal models for TLE (Dashtipour and others, 2003) the presence of persistent basal dendrites in the hilus are reported. In a normal situation basal dendrites are a transient morphological feature of newly born granule cells. In the epileptic hippocampus, hilar basal dendrites remain and receive synaptic input from sprouted mossy fibers (Ribak and others, 2000). Therefore, both hilar basal dendrites and apical dendrites of granule cells contribute to additional recurrent excitatory circuitry via sprouted mossy fibers in the epileptic brain.

Dispersion of granule cells, or the widening of the granule cell layer, is another typical phenomenon in TLE and is correlated with the severity of hippocampal neuronal loss and the extent of mossy fiber sprouting. It remains an unresolved question whether granule cell dispersion is a developmental defect or the result of epileptic seizure activity. Granule cell dispersion is mainly observed in TLE patients who experienced febrile convulsive seizures in early infancy, suggesting an initial precipitating event as a trigger for the development of granule cell dispersion (Houser, 1990; Lurton and others, 1998). The exact mechanism leading to the widening of the granule cell layer has not been elucidated and is still under debate. Possible contributing factors could be local deficits in reelin, a protein that controls neuronal migration (Heinrich and others, 2006) or migration of newly born neurons towards ectopic locations (Jessberger and others, 2005; Parent and others, 2006).

ACUTE AND CHRONIC EPILEPSY MODELS

In order to better understand the mechanisms involved in seizure-initiation, epileptogenesis and spontaneous seizures, different experimental epilepsy models were developed. These models are valuable tools in the ongoing search for new treatments. In general, epilepsy models can be classified in two categories based on whether they display acutely provoked seizures or chronic epilepsy.

In acute seizure models, seizures are evoked by specific stimuli (audiogenic, electrical, chemical). These models are preferably used as an initial screening test for anti-seizure capacities of new potential treatments. Two of the most frequently used acute models are the maximal electroshock (MES) model and the pentylenetetrazol (PTZ) model. The MES model is used to search for compounds with activity against generalized tonic-clonic seizures. Originally in the MES model, a current was delivered via scalp and mouth electrodes in cats (Merritt and Putnam, 1937). Nowadays bilateral transauricular or corneal electrodes are used in the MES model (Walker and others, 2002). The PTZ model involves systemic injection of the convulsant and is used to discover drugs with efficacy against non-convulsive absence or myoclonic seizures (Loscher, 2002).

Chronic epilepsy models more closely resemble the epileptic state in which spontaneous and recurrent seizures occur (Mascott and others, 1994; Loscher, 2002). Many chronic epilepsy models were created specifically to reproduce specific types of human epilepsy, particularly the most common form: TLE (Wieser, 2004). There are chronic TLE models with a genetic predisposition to develop TLE (idiopathic), such as the Ihara epileptic rat displaying neuronal microdysgenesis and gliosis in the hippocampus (Arai and others, 2003). In genetically normal rodents spontaneous seizures, originating from the temporal lobe, can be provoked (symptomatic) by different types of insults such as brain infarction (Kelly and others, 2001; Kelly and others, 2006), traumatic brain damage (Kharatishvili and others, 2006), febrile seizures (Dube and others, 2006), kindling (Michael and others, 1998) or SE. In this study both the kindling and the SE model were used.

THE KINDLING MODEL

Since its discovery by Goddard, the kindling model has been extensively used as a chronic animal model for TLE (Goddard, 1967). The kindling phenomenon is based on the repetitive induction of a focal seizure discharge, which eventually produces a progressive, highly reliable and permanent increase in the epileptic response to the inducing agent. The inducing agent can be chemical (e.g. PTZ) or electrical stimulation, with the latter being most widely used. Electrical stimulation can be focussed to different brain structures such as the amygdala, the hippocampus or the pyriform cortex by using implanted electrodes (Morimoto and others, 2004). The initial seizure response is expressed as a brief focal epileptiform discharge, termed the afterdischarge (AD), evident at the stimulated site and recorded by an implanted electrode. As kindling continues, the AD alters dramatically with an increase in duration, amplitude, spike frequency and an alteration of spike morphology (Racine, 1972).

The intensification of the electrographic response to the stimulation is correlated with an increased severity of behavioural responses. A seizure severity scale called Racine's scale has been developed to score the behavioural responses (Racine, 1972). Racine's scale is widely used in kindling studies stimulating different limbic structures. Racine's grading of convulsive seizures was based on amygdala stimulation: (stage I) immobility, eye closure, ear twitching, twitching of vibrissae, sniffing, facial clonus; (stage II) head nodding associated with more severe facial clonus; (stage III) clonus of one forelimb; (stage IV) bilateral clonus accompanied by rearing without falling; (stage V) generalized tonic-clonic seizures accompanied with rearing and falling. In hippocampal kindling behaviour in the early stages of kindling is different, and therefore a modified version of Racine's scale is generally used: stage I and II seizures represent wet dog shakes, accompanied by head nodding (stage I) and chewing (stage II). Stage III, IV and V correspond to the same behaviour as seen in amygdala kindling. An animal is termed "fully kindled" when electrical stimulation reproducibly evokes generalized tonic-clonic seizures (stage 5 seizures). Once an animal is fully kindled it remains displaying stage 5 seizures in response to stimulation for months or years (Goddard and others, 1969). If kindling stimulation is continued in fully kindled animals they eventually develop spontaneous seizures (Michael and others, 1998).

In the standard kindling protocol, animals receive a kindling stimulation once daily. This allows to fully kindle animals, e.g. rats, within 10 to 15 days dependent on the region which is stimulated. Next to this standard protocol there are also rapid kindling procedures which allow to kindle animals over a much shorter period. In our study, we used the alternate day rapid hippocampal kindling which allows to fully kindle rats with 48 kindling stimulations, spread over four alternating days (Lothman and Williamson, 1994). There are however also rapid kindling protocols in which animals receive 40 kindling stimulations, all in one day (Bengzon and others, 1997; Smith and others, 2005).

Even in rats that demonstrate spontaneous seizures after extensive kindling, gross morphological damage, as observed in patients with HS, are not or only moderately evident (Michael and others, 1998). This confirms that spontaneous seizures can develop without the presence of extensive HS. Based on the controlled induction of epileptogenesis, the kindling model is a very interesting tool to study the role of other events associated with epileptogenesis. However, this model is less appropriate to evaluate spontaneous seizures because it is very labour intensive as a huge number of stimulations have to be administered before spontaneous seizures are evident.

THE STATUS EPILEPTICUS MODEL

The SE model is probably the best characterized chronic TLE model displaying spontaneous seizures. In this model, SE is the initial precipitating insult causing the process of epileptogenesis which eventually results in the occurrence of spontaneous seizures. Next to the occurrence of spontaneous seizures, SE models also display typical histopathological changes in different temporal lobe regions, which closely resemble the pathology seen in human TLE (Tauck and Nadler, 1985; Sutula and others, 1989; Mathern and others, 1993; Mello and others,

1993; Mathern and others, 1995a). Evoking a SE, which is severe enough to cause spontaneous seizures, can be done either by electrical or chemical (kainic acid, pilocarpine) stimulation of various brain regions.

Electrical stimulation can be focussed on the perforant path (Gorter and others, 2001; Mazarati and others, 2002), the ventral hippocampus (Lothman and others, 1989; Bertram and Cornett, 1994), or the amygdala (Nissinen and others, 2000; Brandt and others, 2003). At the beginning of the electrical stimulation the response of the stimulated structure is driven by electrical stimuli. Stimulus-independent epileptiform potentials appear within 15 min. By the end of the stimulation, animals show continuous stimulus-independent discharges called self sustaining status epilepticus (SSSE).

Pilocarpine (PILO) is a muscarinic agonist which induces a SE after systemic injection (Leite and others, 1990; Cavalheiro and others, 1991; Lemos and Cavalheiro, 1995; Arida and others, 1999; Glien and others, 2001; Klitgaard and others, 2002). PILO can be administrated as one large dose (320-400 mg/kg) or as a smaller dose (30 mg/kg), when lithium (127 mg/kg) is given 24 hours before SE. Injection of several low-doses of PILO also efficiently produces SE with a lower mortality compared to higher dose injections of PILO (Glien and others, 2001). Subcutaneous injection of methylscopolamine (1 mg/kg) is normally used to reduce the peripheral effects associated with the autonomic activation caused by PILO (Cavalheiro and others, 1991).

Kainic acid (KA), a structural analogue of glutamic acid, is another frequently used chemoconvulsant and is isolated from the seaweed *Digenea simplex*. KA can be injected systemically as a single bolus or as repeated low doses for the induction of a SE. Systemic injection of a single bolus is often associated with a high mortality rate and a low percentage of rats displaying spontaneous seizures (Leite and others, 1990; Stafstrom and others, 1992). Intraperitoneal injection of repeated low-doses (5 mg/kg) of KA, results in a higher fraction of rats developing spontaneous seizures and less mortality (Hellier and others, 1998).

Both in the systemic PILO and KA models high total doses of both neurotoxins are needed (respectively 20-50 mg/kg and 20-40 mg/kg) with direct toxic effects on the whole brain. By injecting PILO or KA directly into specific brain regions, a SE can be evoked by using much lower doses and limiting direct toxic effects to small brain areas. In a study done by Furtado and coworkers, injection of PILO directly into the hilus of the hippocampus resulted in zero mortality with a high percentage of animals (71%) displaying spontaneous seizures (Furtado Mde and others, 2002). KA has also been directly injected in different brain areas such as in the amygdala (Ben-Ari and others, 1980; Tanaka and others, 1992; Mascott and others, 1994), the ventricle (Sater and Nadler, 1988), the entorhinal cortex (Miettinen and others, 1998) and in the hippocampus (French and others, 1982; Cavalheiro and others, 1983; Leite and others, 1996; Bragin and others, 1999; Bragin and others, 2004; Bragin and others, 2005). Various studies have demonstrated that intracerebral injection of KA and the following SE can provoke the same histopathological changes as seen in human TLE (Leite and others, 1996; Miettinen and others, 1998; Bragin and others, 2004). Other studies have shown that electrophysiological features of epileptogenesis and spontaneous seizures in the intrahippocampal kainic acid

model”resemble those of patients with medically refractory TLE (Bragin and others, 1999; Bragin and others, 2004).

REFERENCES

- Arai M, Amano S, Ryo A, Hada A, Wakatsuki T, Shuda M, Kondoh N, Yamamoto M. 2003. Identification of epilepsy-related genes by gene expression profiling in the hippocampus of genetically epileptic rat. *Brain Res Mol Brain Res* 118(1-2):147-51.
- Arida RM, Scorza FA, Peres CDA, Cavalheiro EA. 1999. The course of untreated seizures in the pilocarpine model of epilepsy. *Epilepsy Research* 34(2-3):99-107.
- Avanzini G, Franceschetti S. 2003. Cellular biology of epileptogenesis. *The Lancet Neurology* 2(1):33-42.
- Babb TL, Kupfer WR, Pretorius JK, Crandall PH, Levesque MF. 1991. Synaptic Reorganization by Mossy Fibers in Human Epileptic Fascia-Dentata. *Neuroscience* 42(2):351-63.
- Babb TL, Lieb JP, Brown WJ, Pretorius J, Crandall PH. 1984. Distribution of pyramidal cell density and hyperexcitability in the epileptic human hippocampal formation. *Epilepsia* 25(6):721-8.
- Ben-Ari Y, Tremblay E, Ottersen OP. 1980. Injections of kainic acid into the amygdaloid complex of the rat: An electrographic, clinical and histological study in relation to the pathology of epilepsy. *Neuroscience* 5(3):515-28.
- Ben-Menachem E. 2002. Vagus-nerve stimulation for the treatment of epilepsy. *Lancet Neurol* 1(8):477-82.
- Bengzon J, Kokaia Z, Elmer E, Nanobashvili A, Kokaia M, Lindvall O. 1997. Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. *Proc Natl Acad Sci U S A* 94(19):10432-7.
- Bertram EH, Cornett JF. 1994. The evolution of a rat model of chronic spontaneous limbic seizures. *Brain Research* 661(1-2):157-62.
- Boon P, Calliauw L, De RJ, Hoksbergen I, Achten E, Thiery E, Caemaert J, De SA, Decoo D. 1994. Clinical and neurophysiological correlations in patients with refractory partial epilepsy and intracranial structural lesions. *Acta Neurochir (Wien)* 128(1-4):68-83.
- Boon P, Vandekerckhove T, Achten E, Thiery E, Goossens L, Vonck K, D'Have M, Van Hoey G, Vanrumste B, Legros B, Defreyne L, De Reuck J. 1999. Epilepsy surgery in Belgium, the experience in Gent. *Acta Neurol Belg* 99(4):256-65.
- Bragin A, Azizyan A, Almajano J, Wilson CL, Engel J, Jr. 2005. Analysis of chronic seizure onsets after intrahippocampal kainic acid injection in freely moving rats. *Epilepsia* 46(10):1592-8.
- Bragin A, Engel J, Jr., Wilson CL, Vinent E, Mathern GW. 1999. Electrophysiologic analysis of a chronic seizure model after unilateral hippocampal KA injection. *Epilepsia* 40(9):1210-21.
- Bragin A, Wilson CL, Almajano J, Mody I, Engel J, Jr. 2004. High-frequency Oscillations after Status Epilepticus: Epileptogenesis and Seizure Genesis. *Epilepsia* 45(9):1017-23.
- Brandt C, Ebert U, Loscher W. 2004. Epilepsy induced by extended amygdala-kindling in rats: lack of clear association between development of spontaneous seizures and neuronal damage. *Epilepsy Res* 62(2-3):135-56.
- Brandt C, Glien M, Potschka H, Volk H, Loscher W. 2003. Epileptogenesis and neuropathology after different types of status epilepticus induced by prolonged electrical stimulation of the basolateral amygdala in rats. *Epilepsy Res* 55(1-2):83-103.
- Brodie MJ, Kwan P. 2002. Staged approach to epilepsy management. *Neurology* 58(8 Suppl 5):S2-S8.
- Buckmaster PS, Zhang GF, Yamawaki R. 2002. Axon sprouting in a model of temporal lobe epilepsy creates a predominantly excitatory feedback circuit. *J Neurosci* 22(15):6650-8.
- Cavalheiro EA, de Feo MR, Mecarelli O, Ricci GF. 1983. Intracortical and intrahippocampal injections of kainic acid in developing rats: an electrographic study. *Electroencephalogr Clin Neurophysiol* 56(5):480-6.
- Cavalheiro EA, Leite JP, Bortolotto ZA, Turski WA, Ikonomidou C, Turski L. 1991. Long-term effects of pilocarpine in rats: structural damage of the brain triggers kindling and spontaneous recurrent seizures. *Epilepsia* 32(6):778-82.
- Cavazos JE, Golarai G, Sutula TP. 1991. Mossy fiber synaptic reorganization induced by kindling: time course of development, progression, and permanence. *J Neurosci* 11(9):2795-803.

- Cavazos JE, Cross DJ. 2006. The role of synaptic reorganization in mesial temporal lobe epilepsy. *Epilepsy & Behavior* 8(3):483-93.
- Clusmann H, Schramm J, Kral T, Helmstaedter C, Ostertun B, Fimmers R, Haun D, Elger CE. 2002. Prognostic factors and outcome after different types of resection for temporal lobe epilepsy. *J Neurosurg* 97(5):1131-41.
- Dashtipour K, Wong AM, Obenaus A, Spigelman I, Ribak CE. 2003. Temporal profile of hilar basal dendrite formation on dentate granule cells after status epilepticus. *Epilepsy Res* 54(2-3):141-51.
- Dube C, Richichi C, Bender RA, Chung G, Litt B, Baram TZ. 2006. Temporal lobe epilepsy after experimental prolonged febrile seizures: prospective analysis. *Brain* 129(4):911-22.
- Engel J, Jr. 1992. Update on surgical treatment of the epilepsies. *Clinical And Experimental Neurology* 29:32-48.
- Engel J. 1996a. Surgery for Seizures. *The New England Journal of Medicine* 334(10):647-53.
- Engel J. 1996b. Introduction to temporal lobe epilepsy. *Epilepsy Research* 26(1):141-50.
- Fisher RS. 1993. Emerging antiepileptic drugs. *Neurology* 43(11 Suppl 5):S12-S20.
- Fisher RS, Boas WvE, Blume W, Elger C, Genton P, Lee P, Engel J. 2005. *Epileptic Seizures and Epilepsy: Definitions Proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE)*. *Epilepsia* 46(4):470-2.
- Foldvary N, Nashold B, Mascha E, Thompson EA, Lee N, McNamara JO, Lewis DV, Luther JS, Friedman AH, Radtke RA. 2000. Seizure outcome after temporal lobectomy for temporal lobe epilepsy: A Kaplan-Meier survival analysis. *Neurology* 54(3):630-4.
- Franck JE, Pokorny J, Kunkel DD, Schwartzkroin PA. 1995. Physiologic and morphologic characteristics of granule cell circuitry in human epileptic hippocampus. *Epilepsia* 36(6):543-58.
- Freeman J, Veggiotti P, Lanzi G, Tagliabue A, Perucca E. 2006. The ketogenic diet: from molecular mechanisms to clinical effects. *Epilepsy Res* 68(2):145-80.
- Freeman JM, Vining EP, Pillas DJ, Pyzik PL, Casey JC, Kelly L, and Millicent. 1998. The Efficacy of the Ketogenic Diet---1998: A Prospective Evaluation of Intervention in 150áChildren. *Pediatrics* 102(6):1358-63.
- French ED, Aldinio C, Schwarcz R. 1982. Intrahippocampal kainic acid, seizures and local neuronal degeneration: relationships assessed in unanesthetized rats. *Neuroscience* 7(10):2525-36.
- French JA, Williamson PD, Thadani VM, Darcey TM, Mattson RH, Spencer SS, Spencer DD. 1993. Characteristics of Medial Temporal-Lobe Epilepsy - .1. Results of History and Physical-Examination. *Annals of Neurology* 34(6):774-80.
- Furtado Mde A, Braga GK, Oliveira JA, Del Vecchio F, Garcia-Cairasco N. 2002. Behavioral, morphologic, and electroencephalographic evaluation of seizures induced by intrahippocampal microinjection of pilocarpine. *Epilepsia* 43(Suppl 5):37-9.
- Glien M, Brandt C, Potschka H, Voigt H, Ebert U, Loscher W. 2001. Repeated low-dose treatment of rats with pilocarpine: low mortality but high proportion of rats developing epilepsy. *Epilepsy Research* 46(2):111-9.
- Goddard GV. 1967. Development of Epileptic Seizures through Brain Stimulation at Low Intensity. *Nature* 214(5092):1020-1.
- Goddard GV, McIntyre DC, Leech CK. 1969. A permanent change in brain function resulting from daily electrical stimulation. *Experimental Neurology* 25(3):295-330.
- Gorter JA, van Vliet EA, Aronica E, Lopes da Silva FH. 2001. Progression of spontaneous seizures after status epilepticus is associated with mossy fibre sprouting and extensive bilateral loss of hilar parvalbumin and somatostatin-immunoreactive neurons. *European Journal of Neuroscience* 13(4):657-69.
- Heinrich C, Nitta N, Flubacher A, Muller M, Fahrner A, Kirsch M, Freiman T, Suzuki F, Depaulis A, Frotscher M, Haas CA. 2006. Reelin Deficiency and Displacement of Mature Neurons, But Not Neurogenesis, Underlie the Formation of Granule Cell Dispersion in the Epileptic Hippocampus. *J Neurosci* 26(17):4701-13.

Hellier JL, Patrylo PR, Buckmaster PS, Dudek FE. 1998. Recurrent spontaneous motor seizures after repeated low-dose systemic treatment with kainate: assessment of a rat model of temporal lobe epilepsy. *Epilepsy Res* 31(1):73-84.

Hermann B, Seidenberg M, Bell B, Rutecki P, Sheth R, Ruggles K, Wendt G, O'Leary D, Magnotta V. 2002. The neurodevelopmental impact of childhood-onset temporal lobe epilepsy on brain structure and function. *Epilepsia* 43(9):1062-71.

Houser CR. 1990. Granule cell dispersion in the dentate gyrus of humans with temporal lobe epilepsy. *Brain Res* 535(2):195-204.

Isokawa M, Levesque MF, Babb TL, Engel J. 1993. Single Mossy Fiber Axonal Systems of Human Dentate Granule Cells Studied in Hippocampal Slices from Patients with Temporal-Lobe Epilepsy. *J Neurosci* 13(4):1511-22.

ILAE. 1981. Proposal for revised clinical and electroencephalographic classification of epileptic seizures. From the Commission on Classification and Terminology of the International League Against Epilepsy. *Epilepsia* 22(4):489-501.

ILAE. 1985. Proposal for classification of epilepsies and epileptic syndromes. Commission on Classification and Terminology of the International League Against Epilepsy. *Epilepsia* 26(3):268-78.

ILAE. 1989. Proposal for revised classification of epilepsies and epileptic syndromes. Commission on Classification and Terminology of the International League Against Epilepsy. *Epilepsia* 30(4):389-99.

Jessberger S, Romer B, Babu H, Kempermann G. 2005. Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. *Exp Neurol* 196(2):342-51.

Jokeit H, Ebner A. 2002. Effects of chronic epilepsy on intellectual functions. *Prog Brain Res* 135:455-63.

Jutila L, Ylinen A, Partanen K, Alafuzoff I, Mervaala E, Partanen J, Vapalahti M, Vainio P, Pitkanen A. 2001. MR volumetry of the entorhinal, perirhinal, and temporopolar cortices in drug-refractory temporal lobe epilepsy. *American Journal of Neuroradiology* 22(8):1490-501.

Kelly KM, Jukkola PI, Kharlamov EA, Downey KL, McBride JW, Strong R, Aronowski J. 2006. Long-term video-EEG recordings following transient unilateral middle cerebral and common carotid artery occlusion in Long-Evans rats. *Experimental Neurology* 201(2):495-506.

Kelly KM, Kharlamov A, Hentosz TM, Kharlamova EA, Williamson JM, Bertram EH, III, Kapur J, Armstrong DM. 2001. Photothrombotic brain infarction results in seizure activity in aging Fischer 344 and Sprague Dawley rats. *Epilepsy Res* 47(3):189-203.

Kharatishvili I, Nissinen JP, McIntosh TK, Pitkanen A. 2006. A model of posttraumatic epilepsy induced by lateral fluid-percussion brain injury in rats. *Neuroscience* 140(2):685-97.

Klitgaard H, Matagne A, Vanneste-Goemaere J, Margineanu DG. 2002. Pilocarpine-induced epileptogenesis in the rat: Impact of initial duration of status epilepticus on electrophysiological and neuropathological alterations. *Epilepsy Research* 51(1-2):93-107.

Kwan P, Brodie MJ. 2001. Effectiveness of first antiepileptic drug. *Epilepsia* 42(10):1255-60.

Leite JP, Babb TL, Pretorius JK, Kuhlman PA, Yeoman KM, Mathern GW. 1996. Neuron loss, mossy fiber sprouting, and interictal spikes after intrahippocampal kainate in developing rats. *Epilepsy Res* 26(1):219-31.

Leite JP, Bortolotto ZA, Cavalheiro EA. 1990. Spontaneous recurrent seizures in rats: an experimental model of partial epilepsy. *Neurosci Biobehav Rev* 14(4):511-7.

Lemos T, Cavalheiro EA. 1995. Suppression of pilocarpine-induced status epilepticus and the late development of epilepsy in rats. *Exp Brain Res* 102(3):423-8.

Lewis DV. 2005. Losing neurons: Selective vulnerability and mesial temporal sclerosis. *Epilepsia* 46:39-44.

Lieb JP, Engel J, Jr., Babb TL. 1987. Interhemispheric propagation time of human hippocampal seizures. I. Relationship to surgical outcome. *Epilepsia* 27(3):286-93.

Longo BM, Mello LE. 1997. Blockade of pilocarpine- or kainate-induced mossy fiber sprouting by cycloheximide does not prevent subsequent epileptogenesis in rats. *Neurosci Lett* 226(3):163-6.

- Longo BM, Mello LE. 1998. Supragranular mossy fiber sprouting is not necessary for spontaneous seizures in the intrahippocampal kainate model of epilepsy in the rat. *Epilepsy Res* 32(1-2):172-82.
- Loscher W. 2002. Animal models of epilepsy for the development of antiepileptogenic and disease-modifying drugs. A comparison of the pharmacology of kindling and post-status epilepticus models of temporal lobe epilepsy. *Epilepsy Res* 50(1-2):105-23.
- Lothman EW, Bertram EH, Bekenstein JW, Perlin JB. 1989. Self-sustaining limbic status epilepticus induced by 'continuous' hippocampal stimulation: electrographic and behavioral characteristics. *Epilepsy Res* 3(2):107-19.
- Lothman EW, Williamson JM. 1994. Closely spaced recurrent hippocampal seizures elicit two types of heightened epileptogenesis: a rapidly developing, transient kindling and a slowly developing, enduring kindling. *Brain Res* 649(1-2):71-84.
- Lurton D, El BB, Sundstrom L, Rougier A. 1998. Granule cell dispersion is correlated with early epileptic events in human temporal lobe epilepsy. *J Neurol Sci* 154(2):133-6.
- Mascott CR, Gotman J, Beaudet A. 1994. Automated EEG monitoring in defining a chronic epilepsy model. *Epilepsia* 35(4):895-902.
- Mathern GW, Babb TL, Armstrong DL. 1999. Hippocampal sclerosis. In: Engel J, Jr., Pedley TA, editors. *Epilepsy: A Comprehensive Textbook*. Philadelphia: Lippincott Williams & Wilkins.
- Mathern GW, Babb TL, Pretorius JK, Leite JP. 1995a. Reactive synaptogenesis and neuron densities for neuropeptide Y, somatostatin, and glutamate decarboxylase immunoreactivity in the epileptogenic human fascia dentata. *J Neurosci* 15(5):3990-4004.
- Mathern GW, Cifuentes F, Leite JP, Pretorius JK, Babb TL. 1993. Hippocampal EEG excitability and chronic spontaneous seizures are associated with aberrant synaptic reorganization in the rat intrahippocampal kainate model. *Electroencephalogr Clin Neurophysiol* 87(5):326-39.
- Mathern GW, Babb TL, Leite JP, Pretorius JK, Yeoman KM, Kuhlman PA. 1996. The pathogenic and progressive features of chronic human hippocampal epilepsy. *Epilepsy Research* 26(1):151-61.
- Mathern GW, Babb TL, Vickrey BG, Melendez M, Pretorius JK. 1995b. The clinical-pathogenic mechanisms of hippocampal neuron loss and surgical outcomes in temporal lobe epilepsy. *Brain* 118(1):105-18.
- Mazarati A, Bragin A, Baldwin R, Shin D, Wilson C, Sankar R, Naylor D, Engel J, Wasterlain CG. 2002. Epileptogenesis after self-sustaining status epilepticus. *Epilepsia* 43(Suppl 5):74-80.
- Mello LE, Cavalheiro EA, Tan AM, Kupfer WR, Pretorius JK, Babb TL, Finch DM. 1993. Circuit mechanisms of seizures in the pilocarpine model of chronic epilepsy: cell loss and mossy fiber sprouting. *Epilepsia* 34(6):985-95.
- Merritt TJ, Putnam HH. 1937. Experimental determination of the anticonvulsant properties of some phenyl derivatives. *Science* 85:525-6.
- Michael M, Holsinger D, Ikeda-Douglas C, Cammisuli S, Ferbinteanu J, DeSouza C, DeSouza S, Fecteau J, Racine RJ, Milgram NW. 1998. Development of spontaneous seizures over extended electrical kindling: I. Electrographic, behavioral, and transfer kindling correlates. *Brain Research* 793(1-2):197-211.
- Miettinen R, Kotti T, Tuunanen J, Toppinen A, Riekkinen S, Halonen T. 1998. Hippocampal damage after injection of kainic acid into the rat entorhinal cortex. *Brain Research* 813(1):9-17.
- Miller LA, McLachlan RS, Bouwer MS, Hudson LP, Munoz DG. 1994. Amygdalar sclerosis: preoperative indicators and outcome after temporal lobectomy. *J Neurol Neurosurg Psychiatry* 57(9):1099-105.
- Morimoto K, Fahnestock M, Racine RJ. 2004. Kindling and status epilepticus models of epilepsy: rewiring the brain. *Prog Neurobiol* 73(1):1-60.
- Nissinen J, Halonen T, Koivisto E, Pitkanen A. 2000. A new model of chronic temporal lobe epilepsy induced by electrical stimulation of the amygdala in rat. *Epilepsy Res* 38(2-3):177-205.
- Panayiotopoulos CP. 2005. Clinical aspects of the diagnosis of epileptic seizures and epileptic syndromes. In: Panayiotopoulos, C.P., editors. *The Epilepsies: Seizures, Syndromes and Management*. first ed. Oxford: Bladon Medical Publishing; p 1-28.

- Parent JM. 2002. The role of seizure-induced neurogenesis in epileptogenesis and brain repair. *Epilepsy Res* 50(1-2):179-89.
- Parent JM, Elliott RC, Pleasure SJ, Barbaro NM, Lowenstein DH. 2006. Aberrant seizure-induced neurogenesis in experimental temporal lobe epilepsy. *Ann Neurol* 59(1):81-91.
- Parrent AG, Blume WT. 1999. Stereotactic amygdalohippocampotomy for the treatment of medial temporal lobe epilepsy. *Epilepsia* 40(10):1408-16.
- Perosa SR, Porcionatto MA, Cukiert A, Martins JR, Amado D, Nader HB, Cavalheiro EA, Leite JP, Naffah-Mazzacoratti MG. 2002. Extracellular matrix components are altered in the hippocampus, cortex, and cerebrospinal fluid of patients with mesial temporal lobe epilepsy. *Epilepsia* 43 Suppl 5:159-61.
- Racine RJ. 1972. Modification of seizure activity by electrical stimulation: II. Motor seizure. *Electroencephalography and Clinical Neurophysiology* 32(3):281-94.
- Regis J, Rey M, Bartolomei F, Vladyka V, Liscak R, Schrottner O, Pendl G. 2004. Gamma knife surgery in mesial temporal lobe epilepsy: a prospective multicenter study. *Epilepsia* 45(5):504-15.
- Ribak CE, Tran PH, Spigelman I, Okazaki MM, Nadler JV. 2000. Status epilepticus-induced hilar basal dendrites on rodent granule cells contribute to recurrent excitatory circuitry. *J Comp Neurol* 428(2):240-53.
- Romcy-Pereira RN, Garcia-Cairasco N. 2003. Hippocampal cell proliferation and epileptogenesis after audiogenic kindling are not accompanied by mossy fiber sprouting or fluoro-jade staining. *Neuroscience* 119(2):533-46.
- Salmenpera T, Kalviainen R, Partanen K, Pitkanen A. 2001. Hippocampal and amygdaloid damage in partial epilepsy - A cross-sectional MRI study of 241 patients. *Epilepsy Research* 46(1):69-82.
- Sater RA, Nadler JV. 1988. On the relation between seizures and brain lesions after intracerebroventricular kainic acid. *Neuroscience Letters* 84(1):73-8.
- Scharfman HE, Sollas AL, Berger RE, Goodman JH. 2003. Electrophysiological evidence of monosynaptic excitatory transmission between granule cells after seizure-induced mossy fiber sprouting. *J Neurophysiol* 90(4):2536-47.
- Smith PD, Mclean KJ, Murphy MA, Turnley AM, Cook MJ. 2005. Seizures, not hippocampal neuronal death, provoke neurogenesis in a mouse rapid electrical amygdala kindling model of seizures. *Neuroscience* 136(2):405-15.
- Spencer DD, Spencer SS. 1994. Hippocampal resections and the use of human tissue in defining temporal lobe epilepsy syndromes. *Hippocampus* 4(3):243-9.
- Spencer SS. 2002. When should temporal-lobe epilepsy be treated surgically? *The Lancet Neurology* 1(6):375-82.
- Srikijvilaikul T, Najm I, Foldvary-Schaefer N, Lineweaver T, Suh JH, Bingaman WE. 2004. Failure of gamma knife radiosurgery for mesial temporal lobe epilepsy: report of five cases. *Neurosurgery* 54(6):1395-402.
- Stafstrom CE, Thompson JL, Holmes GL. 1992. Kainic acid seizures in the developing brain: status epilepticus and spontaneous recurrent seizures. *Brain Res Dev Brain Res* 65(2):227-36.
- Sutula T, Cascino G, Cavazos J, Parada I, Ramirez L. 1989. Mossy Fiber Synaptic Reorganization in the Epileptic Human Temporal-Lobe. *Annals of Neurology* 26(3):321-30.
- Sutula TP, Hagen J, Pitkanen A. 2003. Do epileptic seizures damage the brain? *Curr Opin Neurol* 16(2):189-95.
- Tanaka T, Tanaka S, Fujita T, Takano K, Fukuda H, Sako K, Yonemasu Y. 1992. Experimental complex partial seizures induced by a microinjection of kainic acid into limbic structures. *Progress in Neurobiology* 38(3):317-34.
- Tauack DL, Nadler JV. 1985. Evidence of functional mossy fiber sprouting in hippocampal formation of kainic acid-treated rats. *J Neurosci* 5(4):1016-22.
- Tomson T, Walczak T, Sillanpaa M, Sander JW. 2005. Sudden unexpected death in epilepsy: a review of incidence and risk factors. *Epilepsia* 46 Suppl 11:54-61.

- Velasco F, Carrillo-Ruiz JD, Brito F, Velasco M, Velasco AL, Marquez I, Davis R. 2005. Double-blind, randomized controlled pilot study of bilateral cerebellar stimulation for treatment of intractable motor seizures. *Epilepsia* 46(7):1071-81.
- Vezzani A, Moneta D, Richichi C, Aliprandi M, Burrows SJ, Ravizza T, Perego C, De Simoni MG. 2002. Functional role of inflammatory cytokines and antiinflammatory molecules in seizures and epileptogenesis. *Epilepsia* 43(Suppl 5):30-5.
- von Campe G, Spencer DD, de Lanerolle NC. 1997. Morphology of dentate granule cells in the human epileptogenic hippocampus. *Hippocampus* 7(5):472-88.
- Vonck K, Boon P, Achten E, De RJ, Caemaert J. 2002. Long-term amygdalohippocampal stimulation for refractory temporal lobe epilepsy. *Ann Neurol* 52(5):556-65.
- Vonck K, Thadani VY, Gilbert K, Dedeurwaerdere S, De Groote L, De Herdt V, Goossens L, Gossiaux F, Achten E, Thiery E, Vingerhoets G, van Roost D, Caemaert J, De Reuck J, Roberts D, Williamson P, Boon P. 2004. Vagus nerve stimulation for refractory epilepsy: A transatlantic experience. *Journal of Clinical Neurophysiology* 21(4):283-9.
- Walczak TS. 1995. Neocortical temporal lobe epilepsy: characterizing the syndrome. *Epilepsia* 36(7):633-5.
- Walker MC, White HS, Sander JW. 2002. Disease modification in partial epilepsy. *Brain* 125(Pt 9):1937-50.
- Wiebe S, Blume WT, Girvin JP, Eliasziw M. 2001. A randomized, controlled trial of surgery for temporal-lobe epilepsy. *N Engl J Med* 345(5):311-8.
- Wieser HG. 2004. Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis. *Epilepsia* 45(6):695-714.
- Williamson PD, Engel J, Jr., Munari C. 1999. Anatomic classification of localization-related epilepsies. In: Engel J, Jr., Pedley TA, editors. *Epilepsy: A Comprehensive Textbook*. Philadelphia: Lippincott Williams & Wilkins.
- Wuarin JP, Dudek FE. 1996. Electrographic Seizures and New Recurrent Excitatory Circuits in the Dentate Gyrus of Hippocampal Slices from Kainate-Treated Epileptic Rats. *J Neurosci* 16(14):4438-48.
- Yilmazer-Hanke DM, Wolf HK, Schramm J, Elger CE, Wiestler OD, Blumcke I. 2000. Subregional pathology of the amygdala complex and entorhinal region in surgical specimens from patients with pharmacoresistant temporal lobe epilepsy. *J Neuropathol Exp Neurol* 59(10):907-20.
- Zhang X, Cui S-S, Wallace AE, Hannesson DK, Schmued LC, Saucier DM, Honer WG, Corcoran ME. 2002. Relations between brain pathology and temporal lobe epilepsy. *The journal of neuroscience* 22(14):6052-61.

Chapter 2

Cell Therapy in Neurology

MANUSCRIPT 1

RAEDT R. AND BOON P.

Cell Therapy for Neurological Disorders: A Comprehensive Review.

Acta Neurol. Belg. 2005,**105**, 158-170.

Cell therapy for neurological disorders : a comprehensive review

Robrecht RAEDT and Paul BOON

Laboratory for Clinical and Experimental Neurophysiology, Department of Neurology, Ghent University Hospital, Ghent, Belgium

This manuscript provides a general introduction on different cell therapy strategies that have been implemented and tested in a preclinical and sometimes in a clinical setup. First, different cell types, that have been used or could be used, are critically evaluated. This is followed by an overview of several cell therapy options for different disease mechanisms. Three common neurodegenerative disorders are discussed. First, we show that clinical benefits have been observed in Parkinson's disease, which is probably the most attractive neurodegenerative disease to be treated with cell therapy because of its localized loss of a single cell type. But we also highlight some important side effects of cell therapy in Parkinson's disease. Next, we focus on stroke which involves loss of tissue in focal or diffuse brain regions and we discuss some possible cell therapy options. Finally, we also confer shortly to different cell therapy options for epilepsy. However, we will further elaborate on this in the next chapter of this dissertation.

SUMMARY

Neurodegenerative diseases are characterized by the irreversible loss of neurons involved in networks, important for specific physiological functions. At present, several renewable cell sources stand in line to replace fetal brain cells as potential cell source for transplantation in the damaged brain. Recent developments raise the hope that selective populations of different neuronal phenotypes could be made “on demand”. However, for every potential cell source there are still a lot of questions and drawbacks, which need to be resolved before a cell source could become the standard for clinical neuronal transplantation. The recent finding that the brain responds to damage by increased endogenous neurogenesis could prelude new “neurotrophic therapies”, based on stimulating this endogenous repair. From preclinical studies it is evident that different disease mechanisms require different cell therapy approaches, depending on the underlying factor of the disease, the identity of neuronal systems that are involved and the complexity of networks that are affected. In this review the potential of different cell sources, including the endogenous progenitor cells, are discussed. Also results of preclinical and clinical transplantation studies in three different disease models are critically evaluated.

INTRODUCTION

Current therapies for neurodegenerative diseases provide effective symptomatic relief, particularly in early stages of the disease. However, there are too few therapies, if any, that affect the underlying disease processes. Therefore disease-modifying therapies that halt, slow down or reverse disease progression are sorely needed. Some of the possible treatment options would be: modification of immunological responses, neurotrophic or anti-apoptotic treatment, gene therapy and cell therapy. Replacement of the lost cells seems to be a vital step for functional repair of the brain damage, since in most cases the spared systems cannot replace the function of the lost cells. In contrast to other mammalian tissues the adult mammalian nervous system has weak capabilities for both endogenous cell replacement and pattern repair. The reason for this defective self repair is that adult neuronal cells cannot regenerate after being damaged and that endogenous neural stem cells have only a very limited potential to generate new neuronal cells to replace degenerated neurons. Therefore there is great interest in restoring the damaged nervous system by stimulating endogenous repair or by transplanting new cells into the damaged brain. These cells can be selected on the base of their phenotype, the neurotransmitter they release or by the way they are genetically engineered. Before cell therapy can be a routinely done practice in the clinic, a lot of questions will have to be answered by preclinical research. At this moment different cell sources are tested for their potential to mediate functional repair of brain damage. The goal of this review is to critically evaluate the potential of different candidate cell sources for transplantation. The possibility of stimulating endogenous self repair will be discussed. Three selected neurodegenerative diseases will be presented and the progress and possibilities of cell therapy will be discussed.

CELL SOURCES

FETAL BRAIN TISSUE

Most studies in neurodegenerative diseases have used fetal brain tissue for implantation. Cells are isolated at a time point on which the cells, that have to be implanted, are already fully differentiated in the appropriate cell type. There is a critical time window for the isolation of the population of neurons for implantation. If the relevant neurons are too young, they are not yet differentiated. If they are too old, they have developed extensive connections so that dissection involves axotomy and trauma. This optimal time window, however, varies between different neuronal populations (Dunnett and Bjorklund, 1992). This implies that a lot of fetal brains are necessary to obtain sufficient tissue to be implanted and mostly only one neuronal phenotype can be isolated from one single fetus.

STEM CELLS

The ethical and practical problems around fetal tissue transplantation have led to the search for alternative cell sources. Stem cells seem to be ideal candidates for transplantation. Stem cells are broadly defined as progenitor cells which produce differentiated progeny and are capable to self-renew (Morrison and others, 1997). Stem cells could become an almost unlimited source for the generation of specific neurons. The cell preparations could be standardized and quality-controlled with respect to viability and purity. Different types of stem cells could be used for neuronal transplantation.

NEURAL STEM CELLS

Neural stem cells (NSC) can be isolated from different regions of the embryonic central nervous system (CNS) or from restricted areas in the adult brain. Technical advances in recent years, including the use of bromodeoxyuridine (BrdU) and retroviral reporter mitotic labelling, have shown that the hippocampal dentate gyrus and the forebrain subventricular zone (SVZ), with a rostral migratory stream (RMS) of neuroblasts towards the olfactory bulbs, are germinative regions in which neurogenesis is ongoing throughout life (Cameron and others, 1993; Lois and Alvarez-Buylla, 1994; Lois and others, 1996; van Praag and others, 2002). It is presumed that this ongoing neurogenesis is an integral part of ongoing plasticity in the adult mammalian brain. NSC have been isolated from rodent central nervous system (Palmer and others, 1995; Reynolds and Weiss, 1996; Weiss and others, 1996; Palmer and others, 1997; Shihabuddin and others, 1997; Gritti and others, 1999; Palmer and others, 1999; Temple and Alvarez-Buylla, 1999; Weiss, 1999; Seaberg and van der Kooy, 2002; Galli and others, 2003; Gobbel and others, 2003; Kim and others, 2003) and human brain (Flax and others, 1998; Fricker and others, 1999; Svendsen and others, 1999; Vescovi and others, 1999; Svendsen and Caldwell, 2000; Akiyama and others, 2001; Nunes and others, 2003). NSC are defined by three main characteristics: they can self-renew, give rise to all of the major neural cells types, i.e. neurons, oligodendrocytes and astrocytes (Song and others, 2002) and when transplanted into the brain they are able to

survive, migrate and integrate in a functionally active way (Gage and others, 1995; Flax and others, 1998; Auerbach and others, 2000; Englund and others, 2002). When NSC are transplanted into the damaged brain, they migrate preferentially towards the damaged areas, where they also seem to integrate and replace the lost cells (Flax and others, 1998; Barker and Dunnett, 1999; Yandava and others, 1999; Bjorklund and others, 2002; Dzieczapolski and others, 2003; Pluchino and others, 2003). However, precursors isolated from adult telencephalon and propagated as neurospheres generate disappointingly few neurons, both in transplantation paradigms as well as in differentiating conditions *in vitro* (Fricker and others, 1999; Song and others, 2002). Also the kind of differentiated cell types that they can generate is limited depending upon the developmental stage and region from which they are isolated and the *in vitro* conditions in which they are grown thereafter (Parmar and others, 2002; Hack and others, 2004; Horiguchi and others, 2004).

EMBRYONIC STEM CELLS

Embryonic stem cells are also an attractive cell source for transplantation into the damaged brain. These cells are truly pluripotent and have an unlimited capacity for *in vitro* expansion. The cells can easily be genetically manipulated. Several differentiation protocols have already been developed for differentiation of embryonic stem cells towards neurons and neuronal-restricted precursors (Strubing and others, 1995; Okabe and others, 1996; Li and others, 1998; Mujtaba and others, 1999; Carpenter and others, 2001; Gokhan and Mehler, 2001; O'Shea, 2001; Temple, 2001; Westmoreland and others, 2001; Kim and others, 2002; Wichterle and others, 2002). ES cell-derived neural precursors incorporate into the CNS and differentiate into neurons and glia (Brustle and others, 1997; McDonald and others, 1999; Zhang and others, 2001). Electrophysiological studies have demonstrated that transplanted embryonic stem cell-derived neurons (ESNs) display electrophysiological properties similar to endogenous cells (Kim and others, 2002). Embryonic stem cell-derived glial precursors (ESGPs), have been used successfully for myelin repair (Brustle and others, 1999; Liu and others, 2000) and dye coupling studies showed that the ESGP-derived astrocytes formed gap junctions with each other but also with host astrocytes after transplantation in hippocampal slices (Scheffler and others, 2003).

Although embryonic stem cells seem to have an unrestricted potential to differentiate towards neuroectodermal phenotypes, embryonic stem cells cannot be readily transplanted into the brain. Because of the enormous random *in vitro* differentiation potential of embryonic stem cells, any remaining non-neural (Tabar and Studer, 2002) pluripotent embryonic stem cell could give rise to teratomas upon transplantation, resulting in significant concerns as to the clinical safety of this approach. When ES cells are transplanted into the striatum of an animal model for PD, they differentiate into a significant number of dopamine neurons but the incidence of ES-mediated tumor formation in this study was high (20%) (Bjorklund and others, 2002).

ADULT NON-NEURONAL SOMATIC STEM CELLS

Several recent reports suggest that adult somatic stem cells isolated from non-neuronal tissues may “transdifferentiate” across tissue lineage boundaries, thus offering an accessible source for therapeutic applications and even for neural tissue repair. Human and animal bone marrow (BM) transplantation studies

have shown that donor-derived neurons and glial cells can be found in the brain of the host (Eglitis and Mezey, 1997; Brazelton and others, 2000; Mezey and others, 2000; Mezey and others, 2003). However, the number of these “transdifferentiated” cells is extremely low and recent works have demonstrated that donor BM cells contribute to adult Purkinje neurons through cell fusion (Alvarez-Dolado and others, 2003; Weimann and others, 2003). This is in contrast to another study which demonstrated that human hematopoietic cells could contribute to long term adult human neurogenesis without fusing (Cogle and others, 2004). It seems that fusion as well as transdifferentiation can explain the presence of donor-derived cells in the brain of the recipient. Also purified mesenchymal stem cells, isolated from the bone marrow, seem to be capable of differentiating *in vitro* (Sanchez-Ramos and others, 2000; Woodbury and others, 2000; Black and Woodbury, 2001; Deng and others, 2001; Kohyama and others, 2001; Sanchez-Ramos, 2002; Woodbury and others, 2002; Rismanchi and others, 2003; Dezawa and others, 2004) and *in vivo* (Kopen and others, 1999; Chopp and Li, 2002) towards cells expressing neuronal and glial markers. Expression of neuronal and glial markers, on the contrary, cannot be seen as an absolute proof of neuronal differentiation since it has been demonstrated that undifferentiated mesenchymal stem cells also express markers for neural lineage (Woodbury and others, 2002). Moreover only one study has been able to demonstrate that MSC can differentiate towards neurons displaying appropriate electrophysiological characteristics (Kohyama and others, 2001). In addition to hematopoietic and MSC stem cells, rare pluripotent stem cell subsets have been isolated from BM. Rare cell types, called multipotent adult progenitor cells (MAPC), has been co-isolated with mesenchymal stem cells and is able to differentiate towards cells from the endodermal, mesodermal and ectodermal phenotypes (Jiang and others, 2002). The MAPC is capable of differentiating toward cells with morphological and electrophysiological properties of midbrain neurons (Jiang and others, 2003). Recently a new pluripotent, CD45 negative population from human cord blood, termed unrestricted somatic stem cells (USSCs), has been described (Kogler and others, 2004). It has been demonstrated that these cells can be differentiated towards neuronal cell types. Implantation of these cells in rat brain revealed that human tau-positive neurons persisted in the rat brain for up to 3 months. In this study, though, no electrophysiological experiments were done to confirm that the cells were indeed functionally active neurons. Other cells that display a presumed neurogenic potential are adipose-derived stem cells (Safford and others, 2002) and stem cells derived from the dermis of mammalian skin (Toma and others, 2001).

BIO-ENGINEERED CELLS

Cells can be genetically engineered to overcome problems such as senescence or to induce cells to release neurotrophic or neuromodulating factors. For example, neuroepithelial precursor cells, derived from defined regions and prior to their terminal mitosis, have been infected with a retrovirus carrying a temperature sensitive immortalizing oncogene. When transplanted into the intact brain, most of these cell lines will differentiate towards neurons, astrocytes and oligodendrocytes. They even seem to respond to local microenvironmental cues, since the cells differentiate with morphologies indistinguishable from those of local endogenous neurons (Martinez-Serrano and Bjorklund, 1997; Whittemore and Onifer, 2000). These

immortalized cell lines have been utilized in a variety of *ex vivo* gene therapy experiments, in which they have been genetically modified in order to release different disease modifying molecules. As an example NGF-secreting cells from the HiB5 cell line have been implanted into the adult rat striatum. One week after transplantation a stroke was induced by middle cerebral artery occlusion. The graft prevented striatal degeneration of both projection neurons and cholinergic interneurons (Andsberg and others, 1998). Different other growth factor-, neurotransmitter- or metabolite-releasing immortalized cell lines have been created by genetic engineering. For example, cell lines releasing brain-derived neurotrophic factor (BDNF) (Rubio and others, 1999); neurotrophin 3 (Liu and others, 1999); neurotransmitters, such as GABA (Eaton and others, 1999); or metabolites, such as β -glucuronidase (Snyder and others, 1995) have been developed. Next to these immortalized cell lines other cell sources have been engineered to release disease-modifying substances. Commonly used cell types are fibroblasts (Blesch and others, 2001; Liu and others, 2002; Tobias and others, 2003; Pizzo and others, 2004) and stem cells (Arnhold and others, 2003; Behrstock and Svendsen, 2004; Zhao and others, 2004).

STIMULATING ENDOGENOUS REPAIR

The finding that there is ongoing neurogenesis in the dentate gyrus of the hippocampus and the forebrain SVZ, has led to the idea that stimulation of neurogenesis could enhance endogenous brain repair. There is some suggestion that neurogenesis also exists in other brain regions such as the neocortex (Magavi and others, 2000; Gould and others, 2001), the amygdala (Bernier and others, 2002) and the substantia nigra (Zhao and others, 2003). But these findings are controversial (Kornack and Rakic, 2001; Koketsu and others, 2003). If neurogenesis exists in these regions it is probably at much lower degree or may only be induced after insults (Mohapel and Brundin, 2004). Evidence from *in vivo* studies suggests that specific growth and neurotrophic factors influence neural precursor proliferation in the adult rodent dentate gyrus and SVZ, and in some cases in other brain regions such as striatum, thalamus, hypothalamus, septum and parenchymal regions lining the ventricles. These factors include basic fibroblast growth factor (bFGF), insulin growth factor-1 (IGF-1), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and glial-derived neurotrophic factor (GDNF) (Kuhn and others, 1997; Wagner and others, 1999b; Aberg and others, 2000; Benraiss and others, 2001; Emsley and Hagg, 2003; Schanzer and others, 2004). Several lines of evidence suggest that astrocytes play important roles in the migration, differentiation, integration and survival of neuroblasts derived from the SVZ and the dentate gyrus (Doetsch and others, 1999; Lim and Alvarez-Buylla, 1999; Song and others, 2002; Galli and others, 2003). Because astrocytes are activated by most brain insults, they are most likely also involved in injury-induced neurogenesis.

A lot of work has been done on damaged induced neurogenesis in several models of stroke. Two recent reports indicate that forebrain SVZ neurogenesis increases ipsilateral to the infarct after adult rat transient middle cerebral artery occlusion (Arvidsson and others, 2002; Parent and others, 2002). The neuroblasts generated after stroke form chains closely apposed to astrocytes that extend from the SVZ to the injured striatum although it seems that only a small portion of the newly formed striatal neurons survive. When selective

damage is induced to the hippocampal CA1 region, by inducing transient four vessel ischemia in rats, and subsequently bFGF and EGF are infused for three days in the first week after stroke, 40 % of the CA1 pyramidal neurons are regenerated. The source for the newly generated neurons is demonstrated to be the SVZ in the posterior periventricular region (Nakatomi and others, 2002). Transient global ischemia in young adult macaque monkeys also induces a significant postischemic increase of the number of newly formed cells in the hippocampal dentate gyrus, the subventricular zone of the temporal horn of the lateral ventricle and the temporal neocortex (Tonchev and others, 2003).

CELL THERAPY FOR DIFFERENT NEURONAL DISEASE MECHANISMS

It seems that the potential of cell therapy to restore neuronal damage mostly depends on the complexity of the disease. This ranges from focal cell death of only one neuronal or glial phenotype to more extensive cell death of different neuronal phenotypes throughout the brain. In the next chapter three different disease models with an increasing complexity are presented and the possibilities for developing a cell therapy are evaluated.

REPLACING SINGLE NEURONAL PHENOTYPES: PARKINSON'S DISEASE (PD)

CNS diseases affecting specific neuronal cell populations are Parkinson's disease (PD, loss of striatal dopaminergic neurons), Huntington's disease (HD; loss of GABAergic striatal spiny projection neurons) and amyotrophic lateral sclerosis (ALS, loss of cholinergic motor neurons). These neurodegenerative diseases are the most attractive ones to be treated with cell therapy and therefore a considerable amount of research has been done to investigate the possibilities of repair by cell transplantation. The reader is referred to excellent reviews of these studies (Bjorklund and Lindvall, 2000; Isacson, 2003; Lindvall and others, 2004). In this review only progress in cell therapy for PD will be discussed. In PD there is specific loss of the majority of midbrain dopaminergic neurons projecting towards the striatum. Clinical trials for transplantation of human embryonic mesencephalic tissue into the striatum of patients with severe Parkinson's disease have shown that neuronal replacement can work in the human brain. The grafted neurons survive and reinnervate the striatum for as long as 10 years despite an ongoing disease process (Kordower and others, 1995; Piccini and others, 1999). These open trials have shown that after transplantation dopamine release is elevated and clinical benefit becomes evident (Piccini and others, 2000). A systematic review of 11 studies reporting 95 graft studies was made by (Polgar and others, 2003). Two double blind sham surgery-controlled trials, however, showed no statistically significant improvement in behavioural score. It seems that the outcome of transplantation is dependent on the age of the donor, the severity of the disease (Olanow and others, 2003) and the variation in composition of the graft (Freed and others, 2001). Several studies reported the occurrence of dyskinesias as an important side effect of transplantation, which became troublesome in 7-15% of the grafted patients (Freed and others, 2001; Hagell and others, 2002; Olanow and others, 2003). These rather disappointing results and the occurrence of dyskinesias, next to the limited tissue availability and the wide variation in functional outcome, impelled the search for alternative sources from which large numbers of dopaminergic neurons can be generated. Several recent publications provide a good review of the different studies in which dopaminergic

differentiation of several types of stem cells was investigated (Bjorklund and Lindvall, 2000; Brundin and Hagell, 2001; Lindvall and Hagell, 2002; Lindvall, 2003; Lindvall and McKay, 2003). Functionally active dopaminergic neurons can be generated from mouse (Kim and others, 2002; Morizane and others, 2002) and monkey embryonic stem cells (Kawasaki and others, 2002) and from neural stem cells (NSC) derived from the fetal rodent (Wagner and others, 1999a; Carvey and others, 2001; Yan and others, 2001) and human brain (Storch and others, 2001), using different neuronal differentiation protocols. However, up to now there is only one report describing differentiation of adult neural stem cells towards dopaminergic neurons (Daadi and Weiss, 1999). Also there is little evidence that functional dopaminergic neurons can be obtained from non-neural stem cells. One study described differentiation of mesenchymal stem cells towards functionally active dopaminergic neurons but when these cells were transplanted into the diseased brain they did not differentiate towards neurons (Zhao and others, 2002; Jiang and others, 2003). Dopaminergic neurons derived from stem cells have been transplanted into Parkinson's models and in some cases clear behavioural recovery could be demonstrated (Lindvall, 2003).

CELL THERAPY FOR DISEASES AFFECTING MULTIPLE BRAIN REGIONS AND NEURONAL PHENOTYPES

Probably the most difficult to treat are diseases where transplanted cells should be able to generate multiple phenotypes and reform long distance connections such as in the case of cerebral ischemic insults (Rossi and Cattaneo, 2002) and epilepsy (Grisolia, 2001).

CEREBRAL ISCHEMIC INSULTS

There are two main types of ischemic insults that affect the brain in a specific way. First, cardiac arrest or coronary artery occlusion causes an abrupt and near-total interruption of total cerebral blood flow. This global ischemia causes selective neuronal death of certain vulnerable neuronal populations such as the pyramidal neurons of CA1 hippocampal subregion. In the case of global ischemia, fetal hippocampal CA1 tissue and conditionally immortalized neuroepithelial MHP36 cells have been transplanted into the damaged CA1 region. In the case of transplantation of fetal CA1 tissue behavioural recovery is dependent on the establishment of some afferent and efferent connections. In the case of MHP36 cells there was also a behavioural improvement but only a small portion of the grafted cells displayed neuronal or glial markers. So it remains unclear whether behavioural recovery was caused by restoration of functional connectivity or by secretion of trophic substances (Sinden and others, 1995; Sinden and others, 1997; Virley and others, 1999).

The second type of ischemic insult, stroke, is caused by occlusion of a cerebral artery and leads to irreversible damage in a core region, which is surrounded by a zone of partially reversible injury, the penumbra zone. The majority of cases with stroke in humans are caused by occlusion of the middle cerebral artery, which leads to infarction in the cerebral cortex, basal ganglia and internal capsule. In the only reported clinical trial, neurons generated from the human teratocarcinoma cell line NT-2 have been implanted in the infarcted area of patients, who had experienced a stroke in the basal ganglia. Behavioural improvements were seen in some

patients (Kondziolka and others, 2000) and autopsy in one patient revealed the presence of grafted cell expressing neuronal markers 2 years after grafting (Nelson and others, 2002). Next to this human trial, cells from different origins (fetal cortical and striatal tissue, neural precursor cells, cell lines with neurogenic potential, bone marrow stromal cells) have been transplanted in several stroke models and this in different affected brain regions (cortex, striatum), in the ventricles or intravenously (Savitz and others, 2002; Lindvall and others, 2004). In most cases the transplanted cells survived and a partial behavioural recovery could be seen. However, in few studies there is evidence for a functional integration of these cells into the damaged networks. It is possible that transplantation may enrich the local neural environment through region-specific synaptic connections and trophic factors. Alternatively, grafts may upregulate endogenous recovery mechanisms and induce surviving cells to establish new circuits.

EPILEPSY

Epilepsy has many etiologies, all leading to an imbalance between excitation and inhibition. Unlike in the two other disease mechanisms presented so far, there is no identifiable defect to be restored by cell therapy. Nevertheless, in temporal lobe epilepsy (TLE) there is a common lesion: hippocampal sclerosis (Liu and others, 1995; Blumcke and others, 1999). Hippocampal sclerosis is characterized by a selective loss of hippocampal neurons, axonal sprouting and dense gliosis. However, it is still unproven whether seizures are a cause or an effect of hippocampal sclerosis. Grafting of fetal hippocampal tissue for repair of hippocampal networks in the intracerebral kainic acid model for TLE led to the partial reversal of some of the characteristic anatomopathological changes of hippocampal sclerosis, such as mossy fiber sprouting and loss of GABAergic interneurons. (Shetty and Turner, 1996; Shetty and Turner, 1997a; Shetty and Turner, 1997b; Shetty and others, 2000; Shetty and Turner, 2000; Zaman and others, 2000; Zaman and Shetty, 2001; Zaman and Shetty, 2003). A major caveat in these studies is that the authors have not investigated the influence of transplantation on the occurrence of epileptic seizures (personal communication, Ashok Shetty, 2002). Another transplantation strategy consists of grafting neurotransmitter-releasing cells to modulate network excitability. When GABA-rich fetal striatal tissue is transplanted into the substantia nigra (SNr) of fully amygdala kindled rats this leads to a significant increase in the threshold to electrically evoke focal discharges (after discharge threshold [ADT]) and a significant reduction of seizure severity (Loscher and others, 1998). However, this seizure-suppressing effect was only transient and disappeared over the weeks after transplantation. Noradrenaline-rich locus coeruleus (LC) tissue has been transplanted in the damaged hippocampus of status epilepticus models (Bortolotto and others, 1990). Grafting led to a reduction of the number of spontaneous seizures. But if the transplanted rats were subjected to kindling stimulations approximately eight months after transplantation, no difference in ADT and kindling rate could be demonstrated (Holmes and others, 1991). Next to neurotransmitter rich fetal brain tissue, cells have been engineered to release agents for the inhibition of *in vivo* seizure activity. Thompson *et al.* engineered conditionally immortalized mouse neurons to deliver GABA by driving GAD₆₅ expression under the control of a tetracycline regulatable promoter (Thompson and others, 2000). This cell line has been transplanted into the SNr (Thompson and others, 2000) or the pyriform cortex (Gernert and others, 2002) of

rats prior to kindling. In both cases the transplantation had only weak effects on ADT and kindling rate. These GABA-releasing cells have also been transplanted in the lithium pilocarpine *status epilepticus* model for TLE, which displays spontaneous seizures. The animals were transplanted into the anterior SNr 45-65 days after SE (Thompson and Suchomelova, 2004). Seven to 10 days after transplantation a robust suppression of seizures and the reduction in epileptiform spikes emerged in the group that was transplanted with GABA releasing cells. The evaluation of the seizure suppressant effect of GABA releasing transplants was ended 13 days after transplantation, while it would have been interesting to investigate whether this anticonvulsant effect was long lasting.

Adenosine and its analogues also have powerful antiseizure and neuroprotective activities (Lee and others, 1984; Fredholm, 1997). Therefore baby hamster kidney cells have been engineered to release adenosine in the environment by inactivating the adenosine metabolizing enzyme adenosine kinase (ADK). These adenosine-releasing cells have been encapsulated and transplanted into the ventricles of the rat kindling model (Huber and others, 2001). After transplantation of the cells, behavioural seizure activity was almost completely suppressed during four days after transplantation. This strong protection lasted for three weeks after transplantation after which there was a significant loss of the transplanted cells and the seizure suppressant effect. Embryonic stem cell-derived glial cells have been engineered for adenosine delivery (Fedele and others, 2004). These cells still have to be transplanted into an epilepsy model but it is expected that the survival of these glial cells will be greater compared to the kidney and fibroblast cells, which will probably lead to a more long term seizure suppressant effect.

CONCLUSION

From the evaluation of different cell sources for transplantation it is evident that grafting of fetal cells will not become the standard to treat neurodegenerative diseases because of ethical and practical problems and the high diversity in functional outcome after transplantation. Embryonic and neural stem cells are good alternatives for fetal tissue, given that we learn more about the mechanisms involved in control of cell proliferation and differentiation, neuronal integration and survival. Genetic engineering provides a tool to modify the cells in favour of their survival, integration and their capacity to modify underlying disease mechanisms. Other strategies for reconstruction of damaged networks could be based on the stimulation of endogenous neurogenesis and repair, by means of modulating neurotrophic mechanisms controlling both. Another option could be to combine cell therapy with neurotrophic treatment in order to maximize the recruitment of newborn but also transplanted cells. The cell therapy strategy for a given disease highly depends on the complexity of the disorder. In a disease such as PD, where there is selective loss of dopaminergic neurons, the ultimate goal is to replace the lost cells, repair connectivity and normalize neurotransmitter release. That is why lots of efforts are made to selectively generate dopaminergic cells from different cells sources. In more complex disorders, such as stroke and epilepsy, reconstructive therapy seems to be much further away and therefore other strategies seem to be appropriate in first instance. In stroke, partial recovery

after transplantation sometimes occurs without functional integration of transplanted cells. Therefore neurotrophic responses of both donor and host cells, evoked by the transplantation itself, may play an important role. Transplantation of cells, engineered to secrete neurotrophic factors, could be a first option in the treatment of stroke. In epilepsy most successes can be expected by transplanting cells, which secrete seizure suppressant agents or neurotransmitters, in brain structures that are presumed to play key roles in the generation or spread of epileptic seizures.

REFERENCES

- Aberg MA, Aberg ND, Hedbacker H, Oscarsson J, Eriksson PS. 2000. Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. *J Neurosci* 20(8):2896-903.
- Akiyama Y, Honmou O, Kato T, Uede T, Hashi K, Kocsis JD. 2001. Transplantation of clonal neural precursor cells derived from adult human brain establishes functional peripheral myelin in the rat spinal cord. *Exp Neurol* 167(1):27-39.
- Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A. 2003. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 425(6961):968-73.
- Andsberg G, Kokaia Z, Bjorklund A, Lindvall O, Martinez-Serrano A. 1998. Amelioration of ischaemia-induced neuronal death in the rat striatum by NGF-secreting neural stem cells. *European Journal of Neuroscience* 10(6):2026-36.
- Arnhold S, Hilgers M, Lenartz D, Semkova I, Kochanek S, Voges J, Andressen C, Addicks K. 2003. Neural precursor cells as carriers for a gene therapeutical approach in tumor therapy. *Cell Transplant* 12(8):827-37.
- Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. 2002. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med* 8(9):963-70.
- Auerbach JM, Eiden MV, McKay RD. 2000. Transplanted CNS stem cells form functional synapses in vivo. *European Journal of Neuroscience* 12(5):1696-704.
- Barker RA, Dunnett SB. 1999. Functional integration of neural grafts in Parkinson's disease. *Nat Neurosci* 2(12):1047-8.
- Behrstock S, Svendsen CN. 2004. Combining growth factors, stem cells, and gene therapy for the aging brain. *Ann N Y Acad Sci* 1019:5-14.
- Benraiss A, Chmielnicki E, Lerner K, Roh D, Goldman SA. 2001. Adenoviral brain-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. *J Neurosci* 21(17):6718-31.
- Bernier PJ, Bedard A, Vinet J, Levesque M, Parent A. 2002. Newly generated neurons in the amygdala and adjoining cortex of adult primates. *Proc Natl Acad Sci U S A* 99(17):11464-9.
- Bjorklund A, Lindvall O. 2000. Cell replacement therapies for central nervous system disorders. *Nature Neuroscience* 3(6):537-44.
- Bjorklund LM, Sanchez-Pernate R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlestedt C, Kim KS, Isacson O. 2002. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci U S A* 99(4):2344-9.
- Black IB, Woodbury D. 2001. Adult rat and human bone marrow stromal stem cells differentiate into neurons. *Blood Cells Mol Dis* 27(3):632-6.
- Blesch A, Conner JM, Tuszynski MH. 2001. Modulation of neuronal survival and axonal growth in vivo by tetracycline-regulated neurotrophin expression. *Gene Ther* 8(12):954-60.
- Blumcke I, Beck H, Lie AA, Wiestler OD. 1999. Molecular neuropathology of human mesial temporal lobe epilepsy. *Epilepsy Res* 36(2-3):205-23.
- Bortolotto ZA, Calderazzo L, Cavalheiro EA. 1990. Some evidence that intrahippocampal grafting of noradrenergic neurons suppresses spontaneous seizures in epileptic rats. *Braz J Med Biol Res* 23(12):1267-9.
- Brazelton TR, Rossi FM, Keshet GI, Blau HM. 2000. From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* 290(5497):1775-9.
- Brundin P, Hagell P. 2001. The neurobiology of cell transplantation in Parkinson's disease. *Clinical Neuroscience Research* 1(6):507-20.

- Brustle O, Jones KN, Learish RD, Karram K, Choudhary K, Wiestler OD, Duncan ID, McKay RD. 1999. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* 285(5428):754-6.
- Brustle O, Spiro AC, Karram K, Choudhary K, Okabe S, McKay RD. 1997. In vitro-generated neural precursors participate in mammalian brain development. *Proc Natl Acad Sci U S A* 94(26):14809-14.
- Cameron HA, Woolley CS, McEwen BS, Gould E. 1993. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience* 56(2):337-44.
- Carpenter MK, Inokuma MS, Denham J, Mujtaba T, Chiu CP, Rao MS. 2001. Enrichment of neurons and neural precursors from human embryonic stem cells. *Exp Neurol* 172(2):383-97.
- Carvey PM, Ling ZD, Sortwell CE, Pitzer MR, McGuire SO, Storch A, Collier TJ. 2001. A Clonal Line of Mesencephalic Progenitor Cells Converted to Dopamine Neurons by Hematopoietic Cytokines: A Source of Cells for Transplantation in Parkinson's Disease. *Experimental Neurology* 171(1):98-108.
- Chopp M, Li Y. 2002. Treatment of neural injury with marrow stromal cells. *Lancet Neurol* 1(2):92-100.
- Cogle CR, Yachnis AT, Laywell ED, Zander DS, Wingard JR, Steindler DA, Scott EW. 2004. Bone marrow transdifferentiation in brain after transplantation: a retrospective study. *Lancet* 363(9419):1432-7.
- Daadi MM, Weiss S. 1999. Generation of Tyrosine Hydroxylase-Producing Neurons from Precursors of the Embryonic and Adult Forebrain. *J Neurosci* 19(11):4484-97.
- Deng W, Obrocka M, Fischer I, Prockop DJ. 2001. In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. *Biochem Biophys Res Commun* 282(1):148-52.
- Dezawa M, Kanno H, Hoshino M, Cho H, Matsumoto N, Itokazu Y, Tajima N, Yamada H, Sawada H, Ishikawa H, Mimura T, Kitada M, Suzuki Y, Ide C. 2004. Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J Clin Invest* 113(12):1701-10.
- Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97(6):703-16.
- Dunnett SB, Bjorklund A. 1992. Staging and dissection of rat embryo's. In: Rickwood D., Hames B.D., editors. *Neural transplantation: A practical approach*. 1 ed. New York: Oxford University Press; p 1-19.
- Dziewczapolski G, Lie DC, Ray J, Gage FH, Shults CW. 2003. Survival and differentiation of adult rat-derived neural progenitor cells transplanted to the striatum of hemiparkinsonian rats. *Exp Neurol* 183(2):653-64.
- Eaton MJ, Plunkett JA, Martinez MA, Lopez T, Karmally S, Cejas P, Whittemore SR. 1999. Transplants of neuronal cells bioengineered to synthesize GABA alleviate chronic neuropathic pain. *Cell Transplant* 8(1):87-101.
- Eglitis MA, Mezey E. 1997. Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc Natl Acad Sci U S A* 94(8):4080-5.
- Emsley JG, Hagg T. 2003. Endogenous and exogenous ciliary neurotrophic factor enhances forebrain neurogenesis in adult mice. *Exp Neurol* 183(2):298-310.
- Englund U, Fricker-Gates RA, Lundberg C, Bjorklund A, Wictorin K. 2002. Transplantation of human neural progenitor cells into the neonatal rat brain: extensive migration and differentiation with long-distance axonal projections. *Exp Neurol* 173(1):1-21.
- Fedele DE, Koch P, Scheurer L, Simpson EM, Mohler H, Brustle O, Boison D. 2004. Engineering embryonic stem cell derived glia for adenosine delivery. *Neurosci Lett* 370(2-3):160-5.
- Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billingham LL, Jendoubi M, Sidman RL, Wolfe JH, Kim SU, Snyder EY. 1998. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. *Nat Biotechnol* 16(11):1033-9.
- Fredholm BB. 1997. Adenosine and neuroprotection. *Int Rev Neurobiol* 40:259-80.
- Freed CR, Greene PE, Breeze RE, Tsai WY, DuMouchel W, Kao R, Dillon S, Winfield H, Culver S, Trojanowski JQ, Eidelberg D, Fahn S. 2001. Transplantation of embryonic dopamine neurons for severe Parkinson's disease. *N Engl J Med* 344(10):710-9.

- Fricker RA, Carpenter MK, Winkler C, Greco C, Gates MA, Bjorklund A. 1999. Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. *J Neurosci* 19(14):5990-6005.
- Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, Peterson DA, Suhr ST, Ray J. 1995. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci U S A* 92(25):11879-83.
- Galli R, Gritti A, Bonfanti L, Vescovi AL. 2003. Neural stem cells: an overview. *Circ Res* 92(6):598-608.
- Gernert M, Thompson KW, Loscher W, Tobin AJ. 2002. Genetically engineered GABA-producing cells demonstrate anticonvulsant effects and long-term transgene expression when transplanted into the central piriform cortex of rats. *Exp Neurol* 176(1):183-92.
- Gobbel GT, Choi SJ, Beier S, Niranjana A. 2003. Long-term cultivation of multipotential neural stem cells from adult rat subependyma. *Brain Res* 980(2):221-32.
- Gokhan S, Mehler MF. 2001. Basic and clinical neuroscience applications of embryonic stem cells. *Anat Rec* 265(3):142-56.
- Gould E, Vail N, Wagers M, Gross CG. 2001. Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. *Proc Natl Acad Sci U S A* 98(19):10910-7.
- Grisolia JS. 2001. Stem cell grafting for epilepsy: clinical promise and ethical concerns. *Epilepsy & Behavior* 2:318-23.
- Gritti A, Frolichsthal-Schoeller P, Galli R, Parati EA, Cova L, Pagano SF, Bjornson CR, Vescovi AL. 1999. Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. *J Neurosci* 19(9):3287-97.
- Hack MA, Sugimori M, Lundberg C, Nakafuku M, Gotz M. 2004. Regionalization and fate specification in neurospheres: the role of *Olig2* and *Pax6*. *Molecular and Cellular Neuroscience* 25(4):664-78.
- Hagell P, Piccini P, Bjorklund A, Brundin P, Rehncrona S, Widner H, Crabb L, Pavese N, Oertel WH, Quinn N, Brooks DJ, Lindvall O. 2002. Dyskinesias following neural transplantation in Parkinson's disease. *Nat Neurosci* 5(7):627-8.
- Holmes GL, Thompson JL, Huh K, Holmes C, Carl GF. 1991. Effect of neural transplants on seizure frequency and kindling in immature rats following kainic acid. *Brain Res Dev Brain Res* 64(1-2):47-56.
- Horiguchi S, Takahashi J, Kishi Y, Morizane A, Okamoto Y, Koyanagi M, Tsuji M, Tashiro K, Honjo T, Fujii S, Hashimoto N. 2004. Neural precursor cells derived from human embryonic brain retain regional specificity. *J Neurosci Res* 75(6):817-24.
- Huber A, Padrun V, Deglon N, Aebischer P, Mohler H, Boison D. 2001. Grafts of adenosine-releasing cells suppress seizures in kindling epilepsy. *Proc Natl Acad Sci U S A* 98(13):7611-6.
- Isacson O. 2003. The production and use of cells as therapeutic agents in neurodegenerative diseases. *Lancet Neurology* 2(7):417-24.
- Jiang Y, Henderson D, Blackstad M, Chen A, Miller RF, Verfaillie CM. 2003. Neuroectodermal differentiation from mouse multipotent adult progenitor cells. *Proc Natl Acad Sci U S A* 100 Suppl 1:11854-60.
- Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418(6893):41-9.
- Kawasaki H, Suemori H, Mizuseki K, Watanabe K, Urano F, Ichinose H, Haruta M, Takahashi M, Yoshikawa K, Nishikawa S, Nakatsuji N, Sasai Y. 2002. Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. *Proc Natl Acad Sci U S A* 99(3):1580-5.
- Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-Pernaute R, Bankiewicz K, McKay R. 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418(6893):50-6.

- Kim JY, Koh HC, Lee JY, Chang MY, Kim YC, Chung HY, Son H, Lee YS, Studer L, McKay R, Lee SH. 2003. Dopaminergic neuronal differentiation from rat embryonic neural precursors by Nurr1 overexpression. *Journal of Neurochemistry* 85(6):1443-54.
- Kogler G, Sensken S, Airey JA, Trapp T, Muschen M, Feldhahn N, Liedtke S, Sorg RV, Fischer J, Rosenbaum C, Greschat S, Knipper A, Bender J, Degistirici O, Gao J, Caplan AI, Colletti EJ, Almeida-Porada G, Muller HW, Zanjani E, Wernet P. 2004. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J Exp Med* 200(2):123-35.
- Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, Gojo S, Taga T, Okano H, Hata J, Umezawa A. 2001. Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. *Differentiation* 68(4-5):235-44.
- Koketsu D, Mikami A, Miyamoto Y, Hisatsune T. 2003. Nonrenewal of neurons in the cerebral neocortex of adult macaque monkeys. *J Neurosci* 23(3):937-42.
- Kondziolka D, Wechsler L, Goldstein S, Meltzer C, Thulborn KR, Gebel J, Jannetta P, DeCesare S, Elder EM, McGrogan M, Reitman MA, Bynum L. 2000. Transplantation of cultured human neuronal cells for patients with stroke. *Neurology* 55(4):565-9.
- Kopen GC, Prockop DJ, Phinney DG. 1999. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci U S A* 96(19):10711-6.
- Kordower JH, Freeman TB, Snow BJ, Vingerhoets FJ, Mufson EJ, Sanberg PR, Hauser RA, Smith DA, Nauert GM, Perl DP, . 1995. Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease. *N Engl J Med* 332(17):1118-24.
- Kornack DR, Rakic P. 2001. Cell proliferation without neurogenesis in adult primate neocortex. *Science* 294(5549):2127-30.
- Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH. 1997. Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J Neurosci* 17(15):5820-9.
- Lee KS, Schubert P, Heinemann U. 1984. The anticonvulsive action of adenosine: a postsynaptic, dendritic action by a possible endogenous anticonvulsant. *Brain Res* 321(1):160-4.
- Li M, Pevny L, Lovell-Badge R, Smith A. 1998. Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr Biol* 8(17):971-4.
- Lim DA, Alvarez-Buylla A. 1999. Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. *Proc Natl Acad Sci U S A* 96(13):7526-31.
- Lindvall O. 2003. Stem cells for cell therapy in Parkinson's disease. *Pharmacol Res* 47(4):279-87.
- Lindvall O, Hagell P. 2002. Cell replacement therapy in human neurodegenerative disorders. *Clinical Neuroscience Research* 2(1-2):86-92.
- Lindvall O, Kokaia Z, Martinez-Serrano A. 2004. Stem cell therapy for human neurodegenerative disorders-how to make it work. *Nat Med* 10 Suppl:S42-S50.
- Lindvall O, McKay R. 2003. Brain repair by cell replacement and regeneration. *Proceedings of the National Academy of Sciences of the United States of America* 100(13):7430-1.
- Liu S, Qu Y, Stewart TJ, Howard MJ, Chakraborty S, Holekamp TF, McDonald JW. 2000. Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. *Proc Natl Acad Sci U S A* 97(11):6126-31.
- Liu Y, Himes BT, Murray M, Tessler A, Fischer I. 2002. Grafts of BDNF-producing fibroblasts rescue axotomized rubrospinal neurons and prevent their atrophy. *Exp Neurol* 178(2):150-64.
- Liu Y, Himes BT, Solowska J, Moul J, Chow SY, Park KI, Tessler A, Murray M, Snyder EY, Fischer I. 1999. Intraspinal delivery of neurotrophin-3 using neural stem cells genetically modified by recombinant retrovirus. *Exp Neurol* 158(1):9-26.

- Liu Z, Mikati M, Holmes GL. 1995. Mesial temporal sclerosis: pathogenesis and significance. *Pediatr Neurol* 12(1):5-16.
- Lois C, Alvarez-Buylla A. 1994. Long-distance neuronal migration in the adult mammalian brain. *Science* 264(5162):1145-8.
- Lois C, Garcia-Verdugo JM, Alvarez-Buylla A. 1996. Chain migration of neuronal precursors. *Science* 271(5251):978-81.
- Loscher W, Ebert U, Lehmann H, Rosenthal C, Nikkhah G. 1998. Seizure suppression in kindling epilepsy by grafts of fetal GABAergic neurons in rat substantia nigra. *J Neurosci Res* 51(2):196-209.
- Magavi SS, Leavitt BR, Macklis JD. 2000. Induction of neurogenesis in the neocortex of adult mice. *Nature* 405(6789):951-5.
- Martinez-Serrano A, Bjorklund A. 1997. Immortalized neural progenitor cells for CNS gene transfer and repair. *Trends Neurosci* 20(11):530-8.
- McDonald JW, Liu XZ, Qu Y, Liu S, Mickey SK, Turetsky D, Gottlieb DI, Choi DW. 1999. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat Med* 5(12):1410-2.
- Mezey E, Chandross KJ, Harta G, Maki RA, Mckercher SR. 2000. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* 290(5497):1779-82.
- Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B. 2003. Transplanted bone marrow generates new neurons in human brains. *Proc Natl Acad Sci U S A* 100(3):1364-9.
- Mohapel P, Brundin P. 2004. Harnessing endogenous stem cells to treat neurodegenerative disorders of the basal ganglia. *Parkinsonism Relat Disord* 10(5):259-64.
- Morizane A, Takahashi J, Takagi Y, Sasai Y, Hashimoto N. 2002. Optimal conditions for in vivo induction of dopaminergic neurons from embryonic stem cells through stromal cell-derived inducing activity. *J Neurosci Res* 69(6):934-9.
- Morrison SJ, Shah NM, Anderson DJ. 1997. Regulatory mechanisms in stem cell biology. *Cell* 88(3):287-98.
- Mujtaba T, Piper DR, Kalyani A, Groves AK, Lucero MT, Rao MS. 1999. Lineage-restricted neural precursors can be isolated from both the mouse neural tube and cultured ES cells. *Dev Biol* 214(1):113-27.
- Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M. 2002. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* 110(4):429-41.
- Nelson PT, Kondziolka D, Wechsler L, Goldstein S, Gebel J, DeCesare S, Elder EM, Zhang PJ, Jacobs A, McGrogan M, Lee VM, Trojanowski JQ. 2002. Clonal human (hNT) neuron grafts for stroke therapy: neuropathology in a patient 27 months after implantation. *Am J Pathol* 160(4):1201-6.
- Nunes MC, Roy NS, Keyoung HM, Goodman RR, McKhann G2, Jiang L, Kang J, Nedergaard M, Goldman SA. 2003. Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. *Nat Med* 9(4):439-47.
- O'Shea KS. 2001. Neuronal differentiation of mouse embryonic stem cells: lineage selection and forced differentiation paradigms. *Blood Cells Mol Dis* 27(3):705-12.
- Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD. 1996. Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. *Mech Dev* 59(1):89-102.
- Olanow CW, Goetz CG, Kordower JH, Stoessl AJ, Sossi V, Brin MF, Shannon KM, Nauert GM, Perl DP, Godbold J, Freeman TB. 2003. A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. *Ann Neurol* 54(3):403-14.
- Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH. 1999. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J Neurosci* 19(19):8487-97.
- Palmer TD, Ray J, Gage FH. 1995. FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Mol Cell Neurosci* 6(5):474-86.

- Palmer TD, Takahashi J, Gage FH. 1997. The adult rat hippocampus contains primordial neural stem cells. *Mol Cell Neurosci* 8(6):389-404.
- Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM. 2002. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol* 52(6):802-13.
- Parmar M, Skogh C, Bjorklund A, Campbell K. 2002. Regional Specification of Neurosphere Cultures Derived from Subregions of the Embryonic Telencephalon. *Molecular and Cellular Neuroscience* 21(4):645-56.
- Piccini P, Brooks DJ, Bjorklund A, Gunn RN, Grasby PM, Rimoldi O, Brundin P, Hagell P, Rehncrona S, Widner H, Lindvall O. 1999. Dopamine release from nigral transplants visualized in vivo in a Parkinson's patient. *Nat Neurosci* 2(12):1137-40.
- Piccini P, Lindvall O, Bjorklund A, Brundin P, Hagell P, Ceravolo R, Oertel W, Quinn N, Samuel M, Rehncrona S, Widner H, Brooks DJ. 2000. Delayed recovery of movement-related cortical function in Parkinson's disease after striatal dopaminergic grafts. *Ann Neurol* 48(5):689-95.
- Pizzo DP, Paban V, Coufal NG, Gage FH, Thal LJ. 2004. Long-term production of choline acetyltransferase in the CNS after transplantation of fibroblasts modified with a regulatable vector. *Brain Res Mol Brain Res* 126(1):1-13.
- Pluchino S, Quattrini A, Brambilla E, Gritti A, Salani G, Dina G, Galli R, Del Carro U, Amadio S, Bergami A, Furlan R, Comi G, Vescovi AL, Martino G. 2003. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* 422(6933):688-94.
- Polgar S, Morris ME, Reilly S, Bilney B, Sanberg PR. 2003. Reconstructive neurosurgery for Parkinson's disease: a systematic review and preliminary meta-analysis. *Brain Res Bull* 60(1-2):1-24.
- Reynolds BA, Weiss S. 1996. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* 175(1):1-13.
- Rismanchi N, Floyd CL, Berman RF, Lyeth BG. 2003. Cell death and long-term maintenance of neuron-like state after differentiation of rat bone marrow stromal cells: a comparison of protocols. *Brain Res* 991(1-2):46-55.
- Rossi F, Cattaneo E. 2002. Opinion: neural stem cell therapy for neurological diseases: dreams and reality. *Nat Rev Neurosci* 3(5):401-9.
- Rubio F, Kokaia Z, Arco A, Garcia-Simon M, Snyder E, Lindvall O, Satrustegui J, Martinez-Serrano A. 1999. BDNF gene transfer to the mammalian brain using CNS-derived neural precursors. *Gene Ther* 6(11):1851-66.
- Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM, Rice HE. 2002. Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochem Biophys Res Commun* 294(2):371-9.
- Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. 2000. Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp Neurol* 164(2):247-56.
- Sanchez-Ramos JR. 2002. Neural cells derived from adult bone marrow and umbilical cord blood. *J Neurosci Res* 69(6):880-93.
- Savitz SI, Rosenbaum DM, Dinsmore JH, Wechsler LR, Caplan LR. 2002. Cell transplantation for stroke. *Ann Neurol* 52(3):266-75.
- Schanzer A, Wachs FP, Wilhelm D, Acker T, Cooper-Kuhn C, Beck H, Winkler J, Aigner L, Plate KH, Kuhn HG. 2004. Direct stimulation of adult neural stem cells in vitro and neurogenesis in vivo by vascular endothelial growth factor. *Brain Pathol* 14(3):237-48.
- Scheffler B, Schmandt T, Schroder W, Steinfarz B, Hussein L, Wellmer J, Seifert G, Karram K, Beck H, Blumcke I, Wiestler OD, Steinhauser C, Brustle O. 2003. Functional network integration of embryonic stem cell-derived astrocytes in hippocampal slice cultures. *Development* 130(22):5533-41.
- Seaberg RM, van der Kooy D. 2002. Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. *J Neurosci* 22(5):1784-93.

- Shetty AK, Turner DA. 1996. Development of fetal hippocampal grafts in intact and lesioned hippocampus. *Prog Neurobiol* 50(5-6):597-653.
- Shetty AK, Turner DA. 1997a. Development of long-distance efferent projections from fetal hippocampal grafts depends upon pathway specificity and graft location in kainate-lesioned adult hippocampus. *Neuroscience* 76(4):1205-19.
- Shetty AK, Turner DA. 1997b. Fetal hippocampal cells grafted to kainate-lesioned CA3 region of adult hippocampus suppress aberrant supragranular sprouting of host mossy fibers. *Exp Neurol* 143(2):231-45.
- Shetty AK, Turner DA. 2000. Fetal hippocampal grafts containing CA3 cells restore host hippocampal glutamate decarboxylase-positive interneuron numbers in a rat model of temporal lobe epilepsy. *J Neurosci* 20(23):8788-801.
- Shetty AK, Zaman V, Turner DA. 2000. Pattern of long-distance projections from fetal hippocampal field CA3 and CA1 cell grafts in lesioned CA3 of adult hippocampus follows intrinsic character of respective donor cells. *Neuroscience* 99(2):243-55.
- Shihabuddin LS, Ray J, Gage FH. 1997. FGF-2 is sufficient to isolate progenitors found in the adult mammalian spinal cord. *Exp Neurol* 148(2):577-86.
- Sinden JD, Hodges H, Gray JA. 1995. Neural Transplantation and Recovery of Cognitive Function. *Behavioral and Brain Sciences* 18(1):10-35.
- Sinden JD, Rashid-Doubell F, Kershaw TR, Nelson A, Chadwick A, Jat PS, Noble MD, Hodges H, Gray JA. 1997. Recovery of spatial learning by grafts of a conditionally immortalized hippocampal neuroepithelial cell line into the ischaemia-lesioned hippocampus. *Neuroscience* 81(3):599-608.
- Snyder EY, Taylor RM, Wolfe JH. 1995. Neural progenitor cell engraftment corrects lysosomal storage throughout the MPS VII mouse brain. *Nature* 374(6520):367-70.
- Song HJ, Stevens CF, Gage FH. 2002. Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. *Nat Neurosci* 5(5):438-45.
- Storch A, Paul G, Csete M, Boehm BO, Carvey PM, Kupsch A, Schwarz J. 2001. Long-Term Proliferation and Dopaminergic Differentiation of Human Mesencephalic Neural Precursor Cells. *Experimental Neurology* 170(2):317-25.
- Strubing C, Ahnert-Hilger G, Shan J, Wiedenmann B, Hescheler J, Wobus AM. 1995. Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. *Mech Dev* 53(2):275-87.
- Svendsen CN, Caldwell MA. 2000. Neural stem cells in the developing central nervous system: implications for cell therapy through transplantation. *Prog Brain Res* 127:13-34.
- Svendsen CN, Caldwell MA, Ostenfeld T. 1999. Human neural stem cells: isolation, expansion and transplantation. *Brain Pathol* 9(3):499-513.
- Tabar V, Studer L. 2002. Novel sources of stem cells for brain repair. *Clinical Neuroscience Research* 2(1-2):2-10.
- Temple S. 2001. Stem cell plasticity--building the brain of our dreams. *Nat Rev Neurosci* 2(7):513-20.
- Temple S, Alvarez-Buylla A. 1999. Stem cells in the adult mammalian central nervous system. *Curr Opin Neurobiol* 9(1):135-41.
- Thompson K, Anantharam V, Behrstock S, Bongarzone E, Campagnoni A, Tobin AJ. 2000. Conditionally immortalized cell lines, engineered to produce and release GABA, modulate the development of behavioral seizures. *Exp Neurol* 161(2):481-9.
- Thompson KW, Suchomelova LM. 2004. Transplants of cells engineered to produce GABA suppress spontaneous seizures. *Epilepsia* 45(1):4-12.
- Tobias CA, Shumsky JS, Shibata M, Tuszynski MH, Fischer I, Tessler A, Murray M. 2003. Delayed grafting of BDNF and NT-3 producing fibroblasts into the injured spinal cord stimulates sprouting, partially rescues axotomized red nucleus neurons from loss and atrophy, and provides limited regeneration. *Exp Neurol* 184(1):97-113.

- Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, Miller FD. 2001. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 3(9):778-84.
- Tonchev AB, Yamashima T, Zhao L, Okano HJ, Okano H. 2003. Proliferation of neural and neuronal progenitors after global brain ischemia in young adult macaque monkeys. *Mol Cell Neurosci* 23(2):292-301.
- van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. 2002. Functional neurogenesis in the adult hippocampus. *Nature* 415(6875):1030-4.
- Vescovi AL, Parati EA, Gritti A, Poulin P, Ferrario M, Wanke E, Frolichsthal-Schoeller P, Cova L, Arcellana-Panlilio M, Colombo A, Galli R. 1999. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Exp Neurol* 156(1):71-83.
- Virley D, Ridley RM, Sinden JD, Kershaw TR, Harland S, Rashid T, French S, Sowinski P, Gray JA, Lantos PL, Hodges H. 1999. Primary CA1 and conditionally immortal MHP36 cell grafts restore conditional discrimination learning and recall in marmosets after excitotoxic lesions of the hippocampal CA1 field. *Brain* 122(Pt 12):2321-35.
- Wagner J, Akerud P, Castro DS, Holm PC, Canals JM, Snyder EY, Perlmann T, Arenas E. 1999a. Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes. *Nat Biotechnol* 17(7):653-9.
- Wagner JP, Black IB, DiCicco-Bloom E. 1999b. Stimulation of neonatal and adult brain neurogenesis by subcutaneous injection of basic fibroblast growth factor. *J Neurosci* 19(14):6006-16.
- Weimann JM, Johansson CB, Trejo A, Blau HM. 2003. Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant. *Nat Cell Biol* 5(11):959-66.
- Weiss S. 1999. Pathways for neural stem cell biology and repair. *Nat Biotechnol* 17(9):850-1.
- Weiss S, Dunne C, Hewson J, Wohl C, Wheatley M, Peterson AC, Reynolds BA. 1996. Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J Neurosci* 16(23):7599-609.
- Westmoreland JJ, Hancock CR, Condie BG. 2001. Neuronal development of embryonic stem cells: a model of GABAergic neuron differentiation. *Biochem Biophys Res Commun* 284(3):674-80.
- Whittemore SR, Onifer SM. 2000. Immortalized neural cell lines for CNS transplantation. *Prog Brain Res* 127:49-65.
- Wichterle H, Lieberam I, Porter JA, Jessell TM. 2002. Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110(3):385-97.
- Woodbury D, Reynolds K, Black IB. 2002. Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. *J Neurosci Res* 69(6):908-17.
- Woodbury D, Schwarz EJ, Prockop DJ, Black IB. 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 61(4):364-70.
- Yan J, Studer L, McKay RDG. 2001. Ascorbic acid increases the yield of dopaminergic neurons derived from basic fibroblast growth factor expanded mesencephalic precursors. *Journal of Neurochemistry* 76(1):307-11.
- Yandava BD, Billingham LL, Snyder EY. 1999. "Global" cell replacement is feasible via neural stem cell transplantation: evidence from the dysmyelinated shiverer mouse brain. *Proc Natl Acad Sci U S A* 96(12):7029-34.
- Zaman V, Shetty AK. 2001. Fetal hippocampal CA3 cell grafts transplanted to lesioned CA3 region of the adult hippocampus exhibit long-term survival in a rat model of temporal lobe epilepsy. *Neurobiol Dis* 8(6):942-52.
- Zaman V, Shetty AK. 2003. Fetal hippocampal CA3 cell grafts enriched with fibroblast growth factor-2 exhibit enhanced neuronal integration into the lesioned aging rat hippocampus in a kainate model of temporal lobe epilepsy. *Hippocampus* 13(5):618-32.
- Zaman V, Turner DA, Shetty AK. 2000. Survival of grafted fetal neural cells in kainic acid lesioned CA3 region of adult hippocampus depends upon cell specificity. *Exp Neurol* 161(2):535-61.

Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. 2001. *In vitro* differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol* 19(12):1129-33.

Zhao LR, Duan WM, Reyes M, Keene CD, Verfaillie CM, Low WC. 2002. Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Exp Neurol* 174(1):11-20.

Zhao LX, Zhang J, Cao F, Meng L, Wang DM, Li YH, Nan X, Jiao WC, Zheng M, Xu XH, Pei XT. 2004. Modification of the brain-derived neurotrophic factor gene: a portal to transform mesenchymal stem cells into advantageous engineering cells for neuroregeneration and neuroprotection. *Exp Neurol* 190(2):396-406.

Zhao M, Momma S, Delfani K, Carlen M, Cassidy RM, Johansson CB, Brismar H, Shupliakov O, Frisen J, Janson AM. 2003. Evidence for neurogenesis in the adult mammalian substantia nigra. *Proc Natl Acad Sci U S A* 100(13):7925-30.

ADDENDUM TO MANUSCRIPT 1:

WHY AN ADDENDUM

This addendum is added to the introductory review in order to update the reader with more recent and complete information concerning the isolation, neuronal differentiation and therapeutic potential of different stem cell types (embryonic stem cells, neural stem cells and bone marrow stem cells). First in this addendum a list of criteria is presented which can be used to evaluate the potential of stem cells to differentiate towards neuronal cells. Specific caveats and pitfalls are considered which can lead to false positive results. Next, the latest progress (and criticisms) concerning the differentiation of three stem cell types [embryonic stem cells (ESC), neural stem cells (NSC) and bone marrow (BM)-derived stem cells] is given. Finally, some of the most recent information concerning cell replacement in PD and cerebral ischemic insults are presented. This addendum does not contain additional information concerning cell therapy application for epilepsy as we discuss this more thoroughly in the review, presented in the following chapter, and in the remaining of this dissertation.

CRITERIA FOR NEURONAL DIFFERENTIATION

From the review, presented in the previous chapter, it should be evident that different stem/progenitor cell populations are believed to have the potential to produce different neuronal cell types and to become an extremely important cell source for transplantation in different neurological diseases. Indeed, in the past few years, a huge number of studies have shown that stem or progenitor cells (even of non-neural origin) can be induced to express mature neuronal markers in culture or upon transplantation in the CNS.

When evaluating these studies, one needs to be aware that expression of neuronal markers alone is not sufficient as proof for neuronal differentiation. Mature neuronal genes, for example, are expressed in undifferentiated bone marrow-derived stem cells. This expression is random and non-specific rather than a proof for a neuronal phenotype (Tondreau and others, 2004; Lu and others, 2004). In figure 1, a number of criteria are given which allow demonstrating that cells have the capacity to adopt a neuronal phenotype. The criteria are ordered according to their increasing evidence value.

Expression of neuronal markers can be used to indicate the potential for neuronal differentiation but it is important to verify that this expression occurs in regulated and physiologically relevant manner. First of all, expression of neuronal markers should be detectable both at the gene (mRNA) and protein level. It is thereby also important to verify that the markers are expressed in the proper chronology. Expression of early neural markers (e.g. nestin,sox2) should precede the expression of late neural markers (tau, NF-200). For some neuronal proteins it is also important to verify that they are expressed at appropriate locations within the cell. For example channel-related proteins should be mainly expressed at height of the membrane.

Even when all these criteria on marker expression are fulfilled, it can still not be proven that a cell has the ability to function as a neuron. In order to do so, electrophysiological techniques (e.g. patch clamp studies) are

needed which allow identifying the presence of inward and outward currents, the ability to generate action potentials and the formation of active synapses.

The ultimate, but most difficult to demonstrate, criterion for the ability of a cell type to generate functional neurons is showing that the cells are capable of expressing appropriate neuronal markers, display functional properties and integrate into existing neuronal networks *in vivo*.

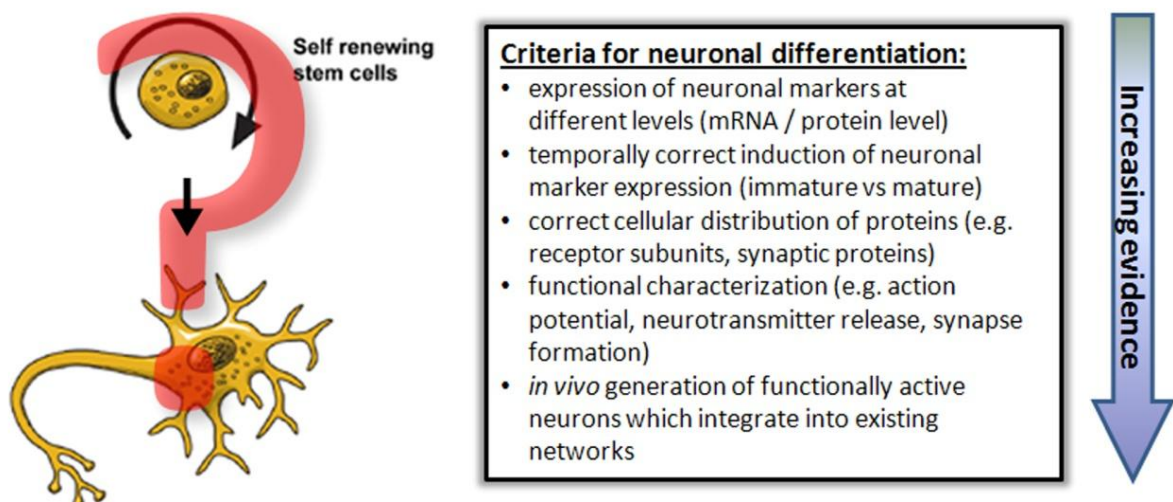


Fig 1.: Criteria which need to be fulfilled to conclude that stem cells are capable of neuronal differentiation

When the *in vivo* generation of neuronal cells is evaluated two important pitfalls need to be considered. The first pitfall concerns the manner of cell labelling. If membrane intercalating dyes or labelled oligonucleotides [5'-bromo-2'-deoxyuridine (BrdU) or tritiated thymidine] are used special care is warranted since those labels can be recycled from dying donor cells to endogenous neuronal cells (Yoon and others, 2005; Burns and others, 2006). In those cases it is necessary to include an additional control group in which labelled dying cells are transplanted. The problem of label transfer can be overcome by genetically engineering the cells to induce the expression of fluorescent proteins or β -galactosidase under a ubiquitous or neural-specific promoter.

Another pitfall that should be taken into account is the possibility of cell fusion. In two reports, transplanted bone marrow-derived cells were reported to spontaneously fuse with endogenous Purkinje neurons (Alvarez-Dolado and others, 2003; Weimann and others, 2003). The product of cell fusion is a cell which has the morphology and properties of a Purkinje neuron and expresses the reported gene (GFP/ β -galactosidase). The occurrence of cell fusion can be identified by performing a sex-mismatched transplantation and using *in situ* hybridization for the sex chromosomes or by using the Cre-lox recombinase system (Wang and others, 2003; Harris and others, 2004). In the latter approach cells, in which ubiquitous expression of reporter gene is inhibited by a floxed inhibitory sequence, are transplanted into mice which ubiquitously express the Cre enzyme. If cell fusion occurs the Cre enzyme excises the floxed inhibitory sequence resulting in the expression of the reporter gene (Harris and others, 2004).

NEURAL DIFFERENTIATION OF EMBRYONIC STEM CELLS

Differentiation of embryonic stem cells (ESC) to neuroepithelial cells is the prerequisite step and gatekeeper toward the generation of different neuronal and glial subclasses. There are three main strategies to convert ESC to neuroepithelial cells *in vitro*: systems based on embryoid-body (EB) formation, neural induction on a stromal feeder layer and protocols based on default differentiation into neural fates.

The first strategy to induce differentiation of ESC toward neuroepithelial cells consists of allowing ESC to aggregate as EBs in suspension culture. EBs contain by default derivatives of all three germ layers but modifications to the protocol have been developed to stimulate neural induction and to select and expand EB-derived neural precursors. These neural progenitors can be further differentiated towards various functional neuronal or glial phenotypes by using patterning, survival or lineage-promoting factors (Bain and others, 1995; Okabe and others, 1996; Brustle and others, 1999; Lee and others, 2000).

One of the standard protocols to stimulate neural differentiation of mouse ESC is based on the exposure of EBs to retinoic acid (RA) (Strubing and others, 1995; Bain and others, 1995). In an alternative EB-based protocol, undifferentiated EBs are exposed to serum-free medium containing insulin, transferrin and selenite (ITS medium). In these minimal growth conditions survival of ESC-derived, nestin expressing neural precursors is selectively favoured while other ESC-derived cells die. The mouse ESC-derived neuronal cells expressed inward and outward currents and action potentials (both evoked and spontaneous) approximately 1 week of differentiation (Strubing and others, 1995). When plated onto organotypic hippocampal slices mouse ESC-derived neuronal precursors demonstrate ionic currents within the first 5 d of culture. ESC-derived neurons also develop action potentials and received synaptic input from host axons (Benninger and others, 2003). Upon transplantation in the developing rat brain the ESC-derived neural precursors migrate into different brain regions, adopt complex morphology, acquire an excitatory or inhibitory neuronal phenotype and form functional synapses with endogenous neurons (Wernig and others, 2004).

To stimulate human EBs to differentiate towards neuroepithelial cells they can be exposed to FGF-2 and subsequently replated to allow the formation of neural tube like structures termed neural rosettes. These human neuroepithelial precursors can be enzymatically separated from the surrounding cell types and replated for further neuronal differentiation. Compared to mouse ESC, human ESC-derived neuronal precursors develop functional properties much more slowly, probably reflecting temporal differences in gestation duration between human and animals. Differentiated human neurons begin firing action potentials only at 7 weeks and repetitive trains of APs are not observed until 10 weeks *in vitro* (Johnson and others, 2007).

The second strategy to promote neuroepithelial differentiation of ESC towards neural cells consist of culturing ESC on a stromal feeder layer. Several stromal cell lines (e.g. PA-6, MS5, S17) exhibit neural inducing properties in co-cultures with ES cells. The molecular nature of this stromal-derived inducing activity remains unknown. Human ESC-derived neural precursors, co-cultured with PA6 cells and transplanted in the developing mouse brain, generate neurons throughout the brain which form functional synapses with surrounding endogenous

neurons (Muotri and others, 2005). At present coculture with PA6 and MS5 cells is the most successful approach to differentiate mouse and human ESCs to dopaminergic neurons (Zhang, 2006).

The third strategy to induce neuronal differentiation is based on the “default hypothesis” which states that in the absence of (BMP) signalling in primitive ectodermal cells will lead to neuronal differentiation. These protocols consist of plating the ESC at low density in minimal conditions without feeders, serum or addition of exogenous growth factors (Trobepe and others, 2001; Ying and others, 2003). When the neural precursors, derived according to this strategy, are exposed to FGF-2 and EGF pure cultures of self-renewing ESC-derived NSC can be obtained. These ESC-derived NSC can be expanded for over 100 passages while retaining the capacity of generating a large proportion of β 3-tubulin expressing neuronal cell types. The expanded cells display a bipolar morphology and express Sox2, Pax6, nestin and RC2 suggestive of a radial glia-like phenotype. During development radial glia function as neural stem cells. In vitro these expanded ESC-derived NSC can be differentiated towards functional neuronal cells capable of firing action potentials. Upon transplantation into the developing embryonic brain and the adult mouse hippocampus the cells express both neural and glial markers (Conti and others, 2005).

The first step towards the use of ESC for transplantation in neurological diseases is the production of highly enriched or even purified populations of specific subclasses of neural precursors. It is thereby extremely important that no undifferentiated ESC remain in the population of cells which will be transplanted. Because of the enormous random *in vitro* differentiation potential of embryonic stem cells, any remaining non-neural pluripotent embryonic stem cell could give rise to teratomas upon transplantation (Bjorklund and others, 2002; Tabar and Studer, 2002). So far, protocols to avoid this teratoma formation are still lacking (Vogel, 2005). Strategies have been developed which use genetic lineage selection to specifically enrich for neural progenitor cells (Li and others, 1998). Also culture protocols (see higher) have been developed which allow the derivation of pure cultures of radial-glia like NSC without the necessity of genetic lineage selection (Conti and others, 2005).

In the standard EB-based neuronal differentiation protocol ESC mainly acquire GABAergic and glutamatergic phenotypes (Studer, 2006). However, in the light of possible therapeutic applications in neurodegenerative diseases great efforts are made to stimulate differentiation of ESC towards specific neuronal phenotypes. For example, there is much scientific interest in selecting/enriching for midbrain dopamine neurons for Parkinson’s disease patients and spinal motor neurons for amyotrophic lateral sclerosis (ALS) patients. One strategy to generate specific neuronal phenotypes is by developing protocols which induce directed differentiation towards the specific neuronal subclasses. These protocols are based on mimicking CNS patterning events during early embryo development. For example, exposure of EBs to RA (caudalizing factor) combined with SHH (ventralizing morphogen) has led to the successful generation of spinal cord motor neurons with high efficiency. These motor neurons develop appropriate transmitter receptors, intrinsic properties which allow the repetitive firing of action potentials and even form functional synapses with muscle fibers in culture (Wichterle

and others, 2002; Miles and others, 2004; Li and others, 2005). For the generation of dopaminergic neurons targeted differentiation relies on the use of FGF-8 and SHH (see further).

An alternative (but possibly complementary) approach to direct *in vitro* differentiation consists of lineage selection based on surface markers or promoter-driven expression of selectable markers. For example lineage selection for Sox2 expressing EB-derived cells after treating EBs with RA has enabled the enrichment of neural progenitor cells (Li and others, 1998). Another example of genetic lineage selection involves the derivation of motor neurons by introducing a plasmid containing eGFP under control of a motor neuron specific promoter (Hb9) in ESC prior to their exposure to RA and SHH (Singh Roy and others, 2005).

DIFFERENTIATION OF BRAIN-DERIVED NEURAL STEM CELLS

As already indicated in the introductory review, NSC have been isolated from a variety of regions of both the developing and adult brain of different mammals, including humans. Two ways of culturing CNS-derived NSC were described. A first technique is to propagate them as neurospheres, which are free-floating spherical clusters containing NSC together with more differentiated neuronal and glial cells. A second technique consists of culturing NSC as monolayers on coated surfaces. There are indications that culturing NSC as neurospheres would favour a gliogenic fate while the monolayer culture would promote a more selective enrichment of NSC (Conti and others, 2005).

In both protocols (neurosphere/monolayer culture) NSC are enriched by selective expansion in response to mitogens, which are in most cases basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). This major disadvantage of this selective expansion technique is that it requires prolonged periods *in vitro*. In order to overcome this prolonged *in vitro* culture, surface antigen-based sorting has been used to selectively isolate NSC (Uchida and others, 2000; Lee and others, 2005). Alternatively genetic lineage selection techniques in which selective markers are placed under the control of NSC-specific promoters (e.g. nestin, musashi1) have proven to be successful (Roy and others, 2000; Keyoung and others, 2001).

In vitro, upon removal of mitogens, NSC are able to differentiate towards astrocytes, oligodendrocytes but also physiologically active neurons which are able to fire action potentials (Liu and others, 1999; Song and others, 2002; Babu and others, 2007). It is still unclear which subclasses of neuronal cells can be derived from brain-derived NSC. It seems that brain-derived NSC are influenced by early brain patterning since neurospheres isolated from different CNS regions express region-specific markers and generate region-specific progeny (Hitoshi and others, 2002; Babu and others, 2007). For example, functional mature dopaminergic neurons have only been generated from NSC isolated from the ventral midbrain (Richardson and others, 2004). However, the differentiation program of NSC can be influenced to some extent by exposing the cells to various growth factors or cytokines (Johe and others, 1996).

NSC can also generate functional neurons *in vivo* but this is most prominent when the cells are transplanted in the developing brain or in neurogenic regions (hippocampus, olfactory bulbs) of the adult brain (Gage and

others, 1995; Flax and others, 1998; Auerbach and others, 2000; Englund and others, 2002). Thus, it seems that the presence of a neurogenic niche is crucial for appropriate neuronal differentiation. From these transplantation studies it is also evident that regional cues can alter the regional identity of brain-derived NSC. For example hippocampus-derived NSC differentiate towards hippocampal granule cells after homotopic transplantation in hippocampus and towards olfactory neurons after heterotopic transplantation in the olfactory bulbs (Suhonen and others, 1996). In non-neurogenic regions (including damaged brain regions) undifferentiated NSC mainly adopt glial cell fates upon transplantation (Gage and others, 1995; Dziewczapolski and others, 2003; Richardson and others, 2005; Conti and others, 2006; Martino and Pluchino, 2006).

One strategy to overcome the inability of NSC to form specific neuronal phenotypes or to generate neurons upon transplantation in non-neurogenic brain regions is to isolate specific neuronal progenitor cells, prior to their terminal differentiation, from developing brain regions and to immortalize them. The latter is frequently done by introducing a temperature sensitive (ts) mutated allele (A58) of the simian virus 40 (SV40) large T-antigen (T-ag) in the cells. Consequently the progenitor cells can be expanded in culture at temperatures below mammalian body temperature (for example 33°C), but upon implantation into a host brain, they stop dividing and differentiate towards mature neural cells (Martinez-Serrano and Bjorklund, 1997). For example the MHP36 neural progenitor cell line was established by dissecting cells from the hippocampal proliferative-zone Anlage at E14, i.e. just before the cells migrate away from the ventricular zone to form the hippocampus (Sinden and others, 1997). MHP36 cells were able to generate site-specific neurons in the non-neurogenic regions of the hippocampus. Additionally MHP36 cells migrate towards lesioned brain areas where they adopt morphologies resembling endogenous neurons.

NEURAL TRANSDIFFERENTIATION OF NON-NEURAL SOMATIC STEM CELLS

As indicated in our review different types of non-neural somatic stem cells (e.g. mesenchymal stem cells, hematopoietic stem cells) are claimed to be capable of generating neural cell types. However, in contrast to neural differentiation of NSC and ESC, this neural “transdifferentiation” potential of non-neural somatic stem cells is subject of much scientific debate.

Most studies claiming neural transdifferentiation have based their conclusions on the expression of neural markers and/or the adoption of neural morphologies. But a variety of studies have demonstrated that non-neural stem cells can express neural markers prior to any differentiation (e.g. MSC, fibroblasts). Furthermore, recent evidence shows the adoption of a neural-like morphology and an upregulation of neural marker expression can be a stress-artefact rather than a true proof for neural differentiation (Neuhuber and others, 2004; Lu and others, 2004; Bertani and others, 2005). Another study evaluated the distribution of neural antigens (e.g. ion channel subunits) in bone marrow cells after “neural transdifferentiation” and found that this was completely different from that seen in adult neurons which probably indicates that the cells cannot function as neurons (Jin and others, 2003).

Only very few studies examined the electrophysiological properties of the bone marrow-derived putative neurons (Kohyama and others, 2001; Hofstetter and others, 2002; Hung and others, 2002; Jiang and others, 2003; Padovan and others, 2003; Dezawa and others, 2004; Wislet-Gendebien and others, 2005). Two reports could not show any electrophysiological evidence for neuronal differentiation despite the expression of neural markers (Hofstetter and others, 2002; Padovan and others, 2003). Two other studies found some voltage dependent currents but could not demonstrate action potentials in the neuron-like cells (Kohyama and others, 2001; Hung and others, 2002).

There have been *in vitro* studies demonstrating that bone marrow-derived stem cells are capable of differentiating towards functionally active neurons, able to fire action potentials. Wislet-Gendebien and colleagues co-cultured nestin-positive MSC with cerebellar granule neurons and found action potentials after 15 days of co-culture. Similar results were obtained when MSC were co-cultured with formalin-fixed granule neurons in the presence of granule cell conditioned medium indicating that cell fusion was not the mechanism behind the neural differentiation (Wislet-Gendebien and others, 2005). Jiang and co-workers co-cultured MAPC, after their exposure to a three-step neuronal induction protocol, with astrocytes for 5 to 12 days and found electrophysiological evidence of neuronal differentiation was evident since action potentials were measured using patch clamp recording (Jiang and others, 2003). In both reports the bone marrow-derived stem cells were not able to fire trains of action potentials nor was there an indication of strong synaptic activity, indicating that the neuronal cells were still immature. Dezawa and colleagues genetically engineered MSC to induce expression of the intracellular Notch domain prior to exposing them to factors which stimulate dopaminergic differentiation. Upon differentiation cells were obtained which were able to generate action potentials, release dopamine in response to depolarization and improve rotational after transplantation in a rat model for Parkinson's disease (Dezawa and others, 2004).

Although several studies have shown that bone marrow-derived stem cells can express neural markers upon transplantation in the brain, none of these studies used electrophysiological techniques to demonstrate that the cells also function as neural cells. Also most of the studies based their conclusion solely on BrdU-labelling and did not perform control experiments where dead cells were transplanted to assure that BrdU was not transferred from dying transplanted cells to endogenously dividing neural precursors (Kopen and others, 1999; Li and others, 2000; Chen and others, 2001; Li and others, 2001; Munoz-Elias and others, 2004; Munoz and others, 2005; Coyne and others, 2006). Furthermore none of these transplantation studies did exclude that cell fusion between donor cells and endogenous neurons/glia was the mechanism behind the presumed neural differentiation.

CELL TRANSPLANTATION FOR PARKINSON'S DISEASE

In the past few years much efforts are made to generate cells which can replace lost midbrain dopaminergic neurons in models of PD. Grafting of poorly differentiated ESC in the striatum of 6-hydroxydopamine (6-OHDA) treated rats gave rise to dopaminergic neurons at the transplant site, restoration of the hemodynamic

response to an amphetamine challenge and functional improvement in motor asymmetry. This indicated that ESC were capable of generating functional dopaminergic neurons capable of integrating in the host striatum. However, in this study it was not confirmed that the cells were donor-derived and also no electrophysiological analysis was done to confirm functional integration of the dopaminergic cells. Also, in 5 out of 25 transplanted rats teratoma-like structures were found at the transplant site (Bjorklund and others, 2002). So, although these results were promising they also indicated that in vitro purification of ESC-derived dopaminergic precursors will be of the greatest importance (see higher). Several strategies to induce directed differentiation of ESC towards dopaminergic neurons have been developed (for reviews see (Conti and others, 2006) and (Zhang, 2006)).

One strategy consists of transducing mouse ESC with the nuclear cell receptor Nurr1, a transcription factor essential for the generation of dopaminergic midbrain neurons. When the Nurr1 mouse ESC were exposed to FGF-8 and SHH in culture, they generated tyrosine hydrolase (TH)-expressing, functionally active midbrain dopaminergic neurons. Transplantation of Nurr1-transduced cells in the striatum of 6-OHDA lesioned rats led to some degree of functional cell replacement with the transplanted neurons innervating the striatum by extending processes up to 2 mm and forming functional synapses. Behavioural studies showed significant reduction in motor asymmetry in the animals that had undergone transplantation. There was no evidence of teratoma formation and the grafts were negative for the proliferation marker Ki-67 (Kim and others, 2002). Recently a protocol has been published which allows the enrichment of dopaminergic neurons to up to 90%. In this protocol Nurr1 transduced ESC were co-cultured with stromal cell lines (PA6) and exposed to FGF-8 and SHH (Kim and others, 2006).

It has been demonstrated that co-culture with stromal cells also stimulates differentiation of human and non-human primate ESC towards midbrain dopaminergic neurons. Perrier and colleagues showed that co-culture of human ESC with MS5 stromal cells and subsequent exposure to SHH and FGF-8 resulted in a high fraction of TH-expressing dopaminergic neurons. Furthermore they found that TH⁺ neurons also expressed engrailed 1 (En-1), a transcription factor which is expressed in midbrain dopamine neurons. The authors also demonstrated the presence of enzymes relating to dopamine metabolism such as aromatic amino acid decarboxylase and vesicular monoamine transporter 2. Furthermore the neurons formed synaptic connections and were capable of generating action potentials in culture (Perrier and others, 2004).

Although the stromal cell-approach is elegant through its simplicity and efficiency, it has the disadvantage that target neural cells are contaminated with stromal cells. Currently major efforts are made to identify the molecular factors which are responsible for this dopaminergic specification and/or differentiation so that a more defined system can be created for the production of dopaminergic neurons. Alternatively, also other differentiation protocols are developed which are not based on the use of stromal cell co-culture systems.

One successful alternative protocol to differentiate human ESC to dopaminergic neurons uses the EB differentiation protocol. After 4 days as free floating EBs, ESC are replated in the presence of FGF-2 to allow the formation neural rosettes. The neural rosettes are then exposed to FGF-8 and SHH to induce dopaminergic

differentiation. In this protocol it is crucial that FGF-8 and SHH are applied at early time points to obtain dopaminergic neurons which co-express the midbrain transcription factor En-1. If factors are applied too late dopaminergic neurons express transcription factors of the forebrain indicating that the ESC have differentiated towards olfactory bulb dopaminergic interneurons (Yan and others, 2005).

It is demonstrated *in vivo* that astrocytes play a crucial role in specifying and maintaining the phenotypes of midbrain dopaminergic neurons. In line with this evidence, a recent study induced dopaminergic differentiation of human ESC in co-culture with mesencephalic astrocytes. The yield of dopaminergic neurons by this approach was approximately 55% of total neurons. After transplantation a robust engraftment of the human ESC-derived dopaminergic neurons was seen and this was correlated with significant behavioural improvement. However, the authors did find continued mitotic activity of neuroepithelial-like tumour cells at the core of the transplant (Roy and others, 2006).

An alternative cell source which could be used for the generation of midbrain dopaminergic neurons are brain-derived NSC. However, up till now, this has been much less successful compared to embryonic stem cells. Human fetal NSC have been transplanted in the striatum of 6-OHDA-lesioned rats but this resulted in a very limited number of donor-derived, TH-expressing dopaminergic neurons 20 weeks after transplantation. A large part of the transplanted NSC had differentiated towards astrocytes (Svendsen and others, 1997). In line with this report several other studies have shown that brain-derived NSC which have been expanded in culture mainly generate glial cells when transplanted in non-neurogenic regions such as the striatum or substantia nigra (Dziewczapolski and others, 2003; Lie and others, 2004; Richardson and others, 2005).

Sanchez-Pernaute and co-workers were more successful in obtaining TH-expressing dopaminergic neurons which were also able to survive after transplantation in the striatum of parkinsonian rats. The authors pretreated short-term expanded, human fetal mesencephalic neural precursors with dibutyryl cyclic adenosine monophosphate and ascorbic acid 2 days prior to transplantation (Sanchez-Pernaute and others, 2001). Six weeks after transplantation robust TH-expression was found in the grafted cells and a significant behavioural improvement. However it is unclear whether the cells would retain the same potential to generate dopaminergic neurons after a more extensive *in vitro* expansion. This is highly unlikely since it was demonstrated for rat fetal mesencephalic neural progenitors that the cells lose their dopaminergic potential after expansion in the presence of FGF-2 (Studer and others, 1998).

Expanded neural precursors can be stimulated to generate functional dopaminergic cells by transduction with Nurr-1 (Kim and others, 2003). Though, these dopaminergic neurons are immature, secrete only minimal amounts of dopamine and do not survive after transplantation in the 6-OHDA lesioned striatum. A recent experiment showed that co-transducing neural precursor cells with Nurr-1 and the neurogenic factor Mash-1 prior to their exposure to neurogenic factors (BDNF and NT-3) promotes the *in vitro* differentiation towards functional and mature dopaminergic neurons. Transplanting these cells in the striatum of parkinsonian rats

lead to the survival of a small fraction of the dopaminergic cells. As a result of the transplantation a significant behavioral improvement was demonstrated (Shim and others, 2007).

One study succeeded in generating cells with characteristics of dopaminergic neurons starting from a non-neuronal cell population. As indicated higher, Dezawa and coworkers transduced human and rat mesenchymal stem cells to induce the expression of the intracellular Notch domain. By exposing the cells to glial derived neurotrophic factor (GDNF) they could be enriched for cells co-expressing TH and midbrain transcription factors (En-1 and Nurr-1). After transplantation into the striatum of parkinsonian rats, cells expressing TH could be detected up to 10 weeks after grafting. As a result of the transplantation there was a higher release of dopamine together with a significant functional improvement (Dezawa and others, 2004). These promising results are not yet confirmed in other laboratories.

Overall, several *in vitro* experiments have succeeded in generating high numbers of dopaminergic neurons (especially from ESC). However, the survival of dopaminergic neurons, transplanted in the brain of PD models, was in the majority of the cases rather disappointing. Also, up till now, none of the transplantation studies was able to demonstrate that stem-cell derived dopaminergic neurons could generate action potentials, form functional synapses and release dopamine in an activity dependant manner (Zhang and others, 2007). One of the possible explanations for these rather disappointing results is that the dopaminergic neurons that are generated *in vitro* may be not the correct type of dopamine neurons. The midbrain neurons that synapse with striatal neurons are located in the substantia nigra. Thus, if dopamine neurons do not possess the nigra dopamine neurons characteristics they may fail to form proper synapses with striatal neurons, which may lead to the death of the transplanted cells (Zhang and others, 2007). Therefore, setting up culture systems which allow the more specific generation nigra dopaminergic neurons could lead to much higher survival rates. Next to the demand for specific subclasses of dopaminergic neurons, the high risk for tumour formation by undifferentiated ESC urges the necessity for more defined differentiation protocols.

Once the right type of dopaminergic progenitors can be generated in purity and abundance, a number of still unresolved issues need to be addressed before the cells can become clinically useful. These include the lack of afferent control of the dopaminergic cells when transplanted in the striatum, their uncontrolled dispersion and the lack of nigrostriatal reconstruction upon alternative transplantation in the substantia nigra. Each of these issues was already considered as problematic in fetal mesencephalic transplant studies.

CELL TRANSPLANTATION FOR STROKE

As already discussed in the review, presented in the previous chapter, cells from a variety of sources such as fetal brain, neuroepithelial cell lines, teratocarcinoma cell lines, bone marrow, umbilical cord, adipose tissue, have been transplanted in the brain of stroke models. For an overview of these studies (including the most recent ones) we refer to an excellent review (Bliss and others, 2007).

In most of the experimental studies on cell transplantation for stroke there was evidence of some behavioural improvement. Many studies showed that cells actively migrate towards the ischemic lesion upon transplantation, probably because of the presence of injury-induced chemoattractants at the lesion site such as stromal-derived factor 1 and monocyte attractant protein 1 (Hill and others, 2004; Newman and others, 2005; Muller and others, 2006; Shen and others, 2007). However only very few studies demonstrated that the transplanted cells had differentiated towards functionally active neurons which had integrated into the network. In their review, Bliss and co-workers, refer to only two studies which could demonstrate that transplanted human or rat NSC had formed synaptic-like structures upon transplantation in the ischemic brain (Toda and others, 2001; Ishibashi and others, 2004). There is also one study which transplanted monkey ESC-derived neural precursor cells in the brain of mice after stroke and found that the cells re-established functional connections with target areas by injecting a retrograde tracer (fluorogold) (Hayashi and others, 2006).

In the majority of the studies survival of the transplanted cells is either too low or the differentiation towards neural cells too minimal to confer the functional improvement to active cell replacement. Instead, in those studies, it is more likely that cell transplantation induced trophic mechanisms which stimulate endogenous recovery and/or protection mechanisms. This hypothesis is further strengthened by the finding that grafting neural progenitor cells contralateral to the lesion can produce the same behavioural recovery as grafting ipsilateral to the lesion (Modo and others, 2002).

Several potential mechanism have been suggested to underlie the trophic effects of cell transplantation in stroke. One frequently suggested hypothesis is that transplanted cells reduce death of endogenous neurons by secretion of trophic factors such as VEGF, FGF, GDNF and BDNF (Johnston and others, 2001; Borlongan and others, 2004; Kurozumi and others, 2005). Alternative mechanisms that could underlie the behavioural recovery could be the stimulation of endogenous neovascularization (Chen and others, 2003b; Taguchi and others, 2004; Jiang and others, 2005; Shyu and others, 2006) or neurogenesis (Chen and others, 2003a; Zhang and others, 2004) through the release of growth factors. Immunomodulation is an alternative mechanism through which cell transplantation could promote behavioural recovery in stroke. There are indications that transplanted NSC can induce apoptosis of T-lymphocytes possibly by the upregulation of membrane expression of certain death receptor ligands (such as FASL, TRAIL and APO3L) (Pluchino and others, 2005). Also NSC are believed to hamper infiltration of blood-born monocytes at the lesion border of ischemic areas where the NSC accumulate (Kelly and others, 2004).

Despite the behavioural improvements described in animal models for stroke and the suggested trophic and immunomodulatory effects of cell transplantation, clinical trials were rather disappointing. In a phase II trial human teratocarcinoma cells (NT2 cell line) were transplanted in 18 chronic basal ganglia stroke patients which were randomized to stereotactic transplantation (n=14) or control (n=4). Only six of the 14 transplanted patients showed slight though non-significant improvement on the motor European Stroke Scale (Bliss and others, 2007). Fetal porcine neural progenitors were transplanted into 5 basal ganglia stroke patients. At 4

years of clinical follow-up only 1 of the 5 treated patients showed significant improvement on the National Institutes of Health Stroke Scale (Savitz and others, 2005).

The disappointing results of these clinical transplantation studies indicate that probably more rational approaches than simply injecting cells into the damaged brain will be required to obtain more satisfactory outcomes. However, based on preclinical research it is very difficult to identify more optimal strategies since transplantation parameters such as delay between lesion and transplantation, cell source, number of transplanted cells, route of cell delivery and target for transplantation varied greatly among experimental studies.

Most probably a cell therapy for stroke will only be of major clinical value when it succeeds to replace dead neurons, remyelinate axons and repair neural circuitries. Therefore a successful cell therapy for stroke will probably require much more complex transplantation paradigms than injecting bone marrow cells intravenously. It will probably be necessary to optimize *in vitro* differentiation strategies to enable the generation of specific neural precursor subtypes. Probably, multiple transplantations will be required at different locations and different time points. Additionally, it is likely that also the brain environment will need to be modified in order to create a niche which supports the integration of new neuronal cells and their sustained survival.

REFERENCES

- Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A. 2003. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 425(6961):968-73.
- Auerbach JM, Eiden MV, McKay RD. 2000. Transplanted CNS stem cells form functional synapses in vivo. *European Journal of Neuroscience* 12(5):1696-704.
- Babu H, Cheung G, Kettenmann H, Palmer TD, Kempermann G. 2007. Enriched monolayer precursor cell cultures from micro-dissected adult mouse dentate gyrus yield functional granule cell-like neurons. *PLoS ONE* 2:e388.e388.
- Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI. 1995. Embryonic Stem Cells Express Neuronal Properties in Vitro. *Developmental Biology* 168(2):342-57.
- Benninger F, Beck H, Wernig M, Tucker KL, Brustle O, Scheffler B. 2003. Functional integration of embryonic stem cell-derived neurons in hippocampal slice cultures. *J Neurosci* 23(18):7075-83.
- Bertani N, Malatesta P, Volpi G, Sonogo P, Perris R. 2005. Neurogenic potential of human mesenchymal stem cells revisited: analysis by immunostaining, time-lapse video and microarray. *J Cell Sci* 118(Pt 17):3925-36.
- Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlestedt C, Kim KS, Isacson O. 2002. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci U S A* 99(4):2344-9.
- Bliss T, Guzman R, Daadi M, Steinberg GK. 2007. Cell Transplantation Therapy for Stroke. *Stroke* 38(2):817-26.
- Borlongan CV, Hadman M, Sanberg CD, Sanberg PR. 2004. Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke. *Stroke* 35(10):2385-9.
- Brustle O, Jones KN, Learish RD, Karram K, Choudhary K, Wiestler OD, Duncan ID, McKay RD. 1999. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* 285(5428):754-6.
- Burns TC, Ortiz-Gonzalez XR, Gutierrez-Perez M, Keene CD, Sharda R, Demorest ZL, Jiang Y, Nelson-Holte M, Soriano M, Nakagawa Y, Luquin MR, Garcia-Verdugo JM, Prosper F, Low WC, Verfaillie CM. 2006. Thymidine Analogs Are Transferred from Prelabeled Donor to Host Cells in the Central Nervous System After Transplantation: A Word of Caution. *Stem Cells* 24(4):1121-7.
- Chen J, Li Y, Katakowski M, Chen X, Wang L, Lu D, Lu M, Gautam SC, Chopp M. 2003a. Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. *J Neurosci Res* 73(6):778-86.
- Chen J, Li Y, Wang L, Lu M, Zhang X, Chopp M. 2001. Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *Journal of the Neurological Sciences* 189(1-2):49-57.
- Chen J, Zhang ZG, Li Y, Wang L, Xu YX, Gautam SC, Lu M, Zhu Z, Chopp M. 2003b. Intravenous Administration of Human Bone Marrow Stromal Cells Induces Angiogenesis in the Ischemic Boundary Zone After Stroke in Rats. *Circulation Research* 92(6):692-9.
- Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying QL, Cattaneo E, Smith A. 2005. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol* 3(9):e283.
- Conti L, Reitano E, Cattaneo E. 2006. Neural stem cell systems: diversities and properties after transplantation in animal models of diseases. *Brain Pathol* 16(2):143-54.
- Coyne TM, Marcus AJ, Woodbury D, Black IB. 2006. Marrow Stromal Cells Transplanted to the Adult Brain Are Rejected by an Inflammatory Response and Transfer Donor Labels to Host Neurons and Glia. *Stem Cells* 24(11):2483-92.
- Dezawa M, Kanno H, Hoshino M, Cho H, Matsumoto N, Itokazu Y, Tajima N, Yamada H, Sawada H, Ishikawa H, Mimura T, Kitada M, Suzuki Y, Ide C. 2004. Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J Clin Invest* 113(12):1701-10.

- Dziewczapolski G, Lie DC, Ray J, Gage FH, Shults CW. 2003. Survival and differentiation of adult rat-derived neural progenitor cells transplanted to the striatum of hemiparkinsonian rats. *Exp Neurol* 183(2):653-64.
- Englund U, Fricker-Gates RA, Lundberg C, Bjorklund A, Wictorin K. 2002. Transplantation of human neural progenitor cells into the neonatal rat brain: extensive migration and differentiation with long-distance axonal projections. *Exp Neurol* 173(1):1-21.
- Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billingham LL, Jendoubi M, Sidman RL, Wolfe JH, Kim SU, Snyder EY. 1998. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. *Nat Biotechnol* 16(11):1033-9.
- Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, Peterson DA, Suhr ST, Ray J. 1995. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci U S A* 92(25):11879-83.
- Harris RG, Herzog EL, Bruscia EM, Grove JE, Van Arnem JS, Krause DS. 2004. Lack of a Fusion Requirement for Development of Bone Marrow-Derived Epithelia. *Science* 305(5680):90-3.
- Hayashi J, Takagi Y, Fukuda H, Imazato T, Nishimura M, Fujimoto M, Takahashi J, Hashimoto N, Nozaki K. 2006. Primate embryonic stem cell-derived neuronal progenitors transplanted into ischemic brain. *J Cereb Blood Flow Metab* 26(7):906-14.
- Hill WD, Hess DC, Martin-Studdard A, Carothers JJ, Zheng J, Hale D, Maeda M, Fagan SC, Carroll JE, Conway SJ. 2004. SDF-1 (CXCL12) is upregulated in the ischemic penumbra following stroke: association with bone marrow cell homing to injury. *J Neuropathol Exp Neurol* 63(1):84-96.
- Hitoshi S, Tropepe V, Ekker M, van der Kooy D. 2002. Neural stem cell lineages are regionally specified, but not committed, within distinct compartments of the developing brain. *Development* 129(1):233-44.
- Hofstetter CP, Schwarz EJ, Hess D, Widenfalk J, El Manira A, Prockop DJ, Olson L. 2002. Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. *PNAS* 99(4):2199-204.
- Hung SC, Cheng H, Pan CY, Tsai MJ, Kao LS, Ma HL. 2002. In vitro differentiation of size-sieved stem cells into electrically active neural cells. *Stem Cells* 20(6):522-9.
- Ishibashi S, Sakaguchi M, Kuroiwa T, Yamasaki M, Kanemura Y, Shizuko I, Shimazaki T, Onodera M, Okano H, Mizusawa H. 2004. Human neural stem/progenitor cells, expanded in long-term neurosphere culture, promote functional recovery after focal ischemia in Mongolian gerbils. *J Neurosci Res* 78(2):215-23.
- Jiang Q, Zhang ZG, Ding GL, Zhang L, Ewing JR, Wang L, Zhang R, Li L, Lu M, Meng H, Arbab AS, Hu J, Li QJ, Pourabdollah ND, Athiraman H, Chopp M. 2005. Investigation of neural progenitor cell induced angiogenesis after embolic stroke in rat using MRI. *NeuroImage* 28(3):698-707.
- Jiang Y, Henderson D, Blackstad M, Chen A, Miller RF, Verfaillie CM. 2003. Neuroectodermal differentiation from mouse multipotent adult progenitor cells. *Proc Natl Acad Sci U S A* 100 Suppl 1:11854-60.
- Jin K, Mao XO, Batteur S, Sun Y, Greenberg DA. 2003. Induction of neuronal markers in bone marrow cells: differential effects of growth factors and patterns of intracellular expression. *Exp Neurol* 184(1):78-89.
- Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RD. 1996. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev* 10(24):3129-40.
- Johnson MA, Weick JP, Pearce RA, Zhang SC. 2007. Functional Neural Development from Human Embryonic Stem Cells: Accelerated Synaptic Activity via Astrocyte Coculture. *J Neurosci* 27(12):3069-77.
- Johnston RE, Ilon-Carter O, Freed WJ, Borlongan CV. 2001. Trophic factor secreting kidney cell lines: in vitro characterization and functional effects following transplantation in ischemic rats. *Brain Research* 900(2):268-76.
- Kelly S, Bliss TM, Shah AK, Sun GH, Ma M, Foo WC, Masel J, Yenari MA, Weissman IL, Uchida N, Palmer T, Steinberg GK. 2004. Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. *PNAS* 101(32):11839-44.
- Keyoung HM, Roy NS, Benraiss A, Louissaint A, Jr., Suzuki A, Hashimoto M, Rashbaum WK, Okano H, Goldman SA. 2001. High-yield selection and extraction of two promoter-defined phenotypes of neural stem cells from the fetal human brain. *Nat Biotechnol* 19(9):843-50.

- Kim DW, Chung S, Hwang M, Ferree A, Tsai HC, Park JJ, Chung S, Nam TS, Kang UJ, Isacson O, Kim KS. 2006. Stromal Cell-Derived Inducing Activity, Nurr1, and Signaling Molecules Synergistically Induce Dopaminergic Neurons from Mouse Embryonic Stem Cells. *Stem Cells* 24(3):557-67.
- Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-Pernaute R, Bankiewicz K, McKay R. 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418(6893):50-6.
- Kim JY, Koh HC, Lee JY, Chang MY, Kim YC, Chung HY, Son H, Lee YS, Studer L, McKay R, Lee SH. 2003. Dopaminergic neuronal differentiation from rat embryonic neural precursors by Nurr1 overexpression. *J Neurochem* 85(6):1443-54.
- Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, Gojo S, Taga T, Okano H, Hata J, Umezawa A. 2001. Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. *Differentiation* 68(4-5):235-44.
- Kopen GC, Prockop DJ, Phinney DG. 1999. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci U S A* 96(19):10711-6.
- Kurozumi K, Nakamura K, Tamiya T, Kawano Y, Ishii K, Kobune M, Hirai S, Uchida H, Sasaki K, Ito Y, Kato K, Honmou O, Houkin K, Date I, Hamada H. 2005. Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model. *Mol Ther* 11(1):96-104.
- Lee A, Kessler JD, Read TA, Kaiser C, Corbeil D, Huttner WB, Johnson JE, Wechsler-Reya RJ. 2005. Isolation of neural stem cells from the postnatal cerebellum. *Nat Neurosci* 8(6):723-9.
- Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD. 2000. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol* 18(6):675-9.
- Li M, Pevny L, Lovell-Badge R, Smith A. 1998. Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr Biol* 8(17):971-4.
- Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, Zhang SC. 2005. Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol* 23(2):215-21.
- Li Y, Chen J, Wang L, Zhang L, Lu M, Chopp M. 2001. Intracerebral transplantation of bone marrow stromal cells in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *Neuroscience Letters* 316(2):67-70.
- Li Y, Chopp M, Chen J, Wang L, Gautam SC, Xu YX, Zhang Z. 2000. Intrastriatal Transplantation of Bone Marrow Nonhematopoietic Cells Improves Functional Recovery After Stroke in Adult Mice. *J Cereb Blood Flow Metab* 20(9):1311-9.
- Lie DC, Song H, Colamarino SA, Ming GL, Gage FH. 2004. Neurogenesis in the adult brain: new strategies for central nervous system diseases. *Annu Rev Pharmacol Toxicol* 44:399-421.
- Liu RH, Morassutti DJ, Whittemore SR, Sosnowski JS, Magnuson DSK. 1999. Electrophysiological Properties of Mitogen-Expanded Adult Rat Spinal Cord and Subventricular Zone Neural Precursor Cells. *Experimental Neurology* 158(1):143-54.
- Lu P, Blesch A, Tuszynski MH. 2004. Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? *J Neurosci Res* 77(2):174-91.
- Martinez-Serrano A, Bjorklund A. 1997. Immortalized neural progenitor cells for CNS gene transfer and repair. *Trends Neurosci* 20(11):530-8.
- Martino G, Pluchino S. 2006. The therapeutic potential of neural stem cells. *Nat Rev Neurosci* 7(5):395-406.
- Miles GB, Yohn DC, Wichterle H, Jessell TM, Rafuse VF, Brownstone RM. 2004. Functional Properties of Motoneurons Derived from Mouse Embryonic Stem Cells. *J Neurosci* 24(36):7848-58.
- Modo M, Stroemer RP, Tang E, Patel S, Hodges H. 2002. Effects of Implantation Site of Stem Cell Grafts on Behavioral Recovery From Stroke Damage. *Stroke* 33(9):2270-8.
- Muller FJ, Snyder EY, Loring JF. 2006. Gene therapy: can neural stem cells deliver? *Nat Rev Neurosci* 7(1):75-84.

- Munoz JR, Stoutenger BR, Robinson AP, Spees JL, Prockop DJ. 2005. Human stem/progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice. *PNAS* 102(50):18171-6.
- Munoz-Elias G, Marcus AJ, Coyne TM, Woodbury D, Black IB. 2004. Adult bone marrow stromal cells in the embryonic brain: Engraftment, migration, differentiation, and long-term survival. *J Neurosci* 24(19):4585-95.
- Muotri AR, Nakashima K, Toni N, Sandler VM, Gage FH. 2005. Development of functional human embryonic stem cell-derived neurons in mouse brain. *PNAS* 102(51):18644-8.
- Neuhuber B, Gallo G, Howard L, Kostura L, Mackay A, Fischer I. 2004. Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. *J Neurosci Res* 77(2):192-204.
- Newman MB, Willing AE, Manresa JJ, vis-Sanberg C, Sanberg PR. 2005. Stroke-induced migration of human umbilical cord blood cells: time course and cytokines. *Stem Cells Dev* 14(5):576-86.
- Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD. 1996. Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. *Mech Dev* 59(1):89-102.
- Padovan CS, Jahn K, Birnbaum T, Reich P, Sostak P, Strupp M, Straube A. 2003. Expression of neuronal markers in differentiated marrow stromal cells and CD133+ stem-like cells 40. *Cell Transplant* 12(8):839-48.
- Perrier AL, Tabar V, Barberi T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L. 2004. From the Cover: Derivation of midbrain dopamine neurons from human embryonic stem cells. *PNAS* 101(34):12543-8.
- Pluchino S, Zanotti L, Rossi B, Brambilla E, Ottoboni L, Salani G, Martinello M, Cattalini A, Bergami A, Furlan R, Comi G, Constantin G, Martino G. 2005. Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. *Nature* 436(7048):266-71.
- Richardson RM, Fillmore HL, Holloway KL, Broaddus WC. 2004. Progress in cerebral transplantation of expanded neuronal stem cells. *J Neurosurg* 100(4):659-71.
- Richardson RM, Broaddus WC, Holloway KL, Fillmore HL. 2005. Grafts of adult subependymal zone neuronal progenitor cells rescue hemiparkinsonian behavioral decline. *Brain Research* 1032(1-2):11-22.
- Roy NS, Benraiss A, Wang S, Fraser RA, Goodman R, Couldwell WT, Nedergaard M, Kawaguchi A, Okano H, Goldman SA. 2000. Promoter-targeted selection and isolation of neural progenitor cells from the adult human ventricular zone. *J Neurosci Res* 59(3):321-31.
- Roy NS, Cleren C, Singh SK, Yang L, Beal MF, Goldman SA. 2006. Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes. *Nat Med* 12(11):1259-68.
- Sanchez-Pernaute R, Studer L, Bankiewicz KS, Major EO, McKay RD. 2001. In vitro generation and transplantation of precursor-derived human dopamine neurons. *J Neurosci Res* 65(4):284-8.
- Savitz SI, Dinsmore J, Wu J, Henderson GV, Stieg P, Caplan LR. 2005. Neurotransplantation of fetal porcine cells in patients with basal ganglia infarcts: a preliminary safety and feasibility study. *Cerebrovasc Dis* 20(2):101-7.
- Shen LH, Li Y, Chen J, Zacharek A, Gao Q, Kapke A, Lu M, Raginski K, Vanguri P, Smith A, Chopp M. 2007. Therapeutic benefit of bone marrow stromal cells administered 1 month after stroke. *J Cereb Blood Flow Metab* 27(1):6-13.
- Shim JW, Park CH, Bae YC, Bae JY, Chung S, Chang MY, Koh HC, Lee HS, Hwang SJ, Lee KH, Lee YS, Choi CY, Lee SH. 2007. Generation of Functional Dopamine Neurons from Neural Precursor Cells Isolated from the Subventricular Zone and White Matter of the Adult Rat Brain Using Nurr1 Overexpression. *Stem Cells* 25(5):1252-62.
- Shyu WC, Lin SZ, Chiang MF, Su CY, Li H. 2006. Intracerebral Peripheral Blood Stem Cell (CD34+) Implantation Induces Neuroplasticity by Enhancing beta1 Integrin-Mediated Angiogenesis in Chronic Stroke Rats. *J Neurosci* 26(13):3444-53.

- Sinden JD, Rashid-Doubell F, Kershaw TR, Nelson A, Chadwick A, Jat PS, Noble MD, Hodges H, Gray JA. 1997. Recovery of spatial learning by grafts of a conditionally immortalized hippocampal neuroepithelial cell line into the ischaemia-lesioned hippocampus. *Neuroscience* 81(3):599-608.
- Singh Roy N, Nakano T, Xuing L, Kang J, Nedergaard M, Goldman SA. 2005. Enhancer-specified GFP-based FACS purification of human spinal motor neurons from embryonic stem cells. *Experimental Neurology* 196(2):224-34.
- Song HJ, Stevens CF, Gage FH. 2002. Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. *Nat Neurosci* 5(5):438-45.
- Strubing C, Ahnert-Hilger G, Shan J, Wiedenmann B, Hescheler J, Wobus AM. 1995. Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. *Mech Dev* 53(2):275-87.
- Studer L. 2006. The Nervous System. In: Lanza R, Gearhart J, Hogan B, Melton D, Pedersen R, Thomson J, E.Donnall T, West M, editors. *Essentials of stem cell biology*. London: Elsevier Inc.; p 149-56.
- Studer L, Tabar V, McKay RD. 1998. Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. *Nat Neurosci* 1(4):290-5.
- Suhonen JO, Peterson DA, Ray J, Gage FH. 1996. Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. *Nature* 383(6601):624-7.
- Svendsen CN, Caldwell MA, Shen J, ter Borg MG, Rosser AE, Tyers P, Karmiol S, Dunnett SB. 1997. Long-Term Survival of Human Central Nervous System Progenitor Cells Transplanted into a Rat Model of Parkinson's Disease. *Experimental Neurology* 148(1):135-46.
- Tabar V, Studer L. 2002. Novel sources of stem cells for brain repair. *Clinical Neuroscience Research* 2(1-2):2-10.
- Taguchi A, Soma T, Tanaka H, Kanda T, Nishimura H, Yoshikawa H, Tsukamoto Y, Iso H, Fujimori Y, Stern DM, Naritomi H, Matsuyama T. 2004. Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. *J Clin Invest* 114(3):330-8.
- Toda H, Takahashi J, Iwakami N, Kimura T, Hoki S, Mozumi-Kitamura K, Ono S, Hashimoto N. 2001. Grafting neural stem cells improved the impaired spatial recognition in ischemic rats. *Neurosci Lett* 316(1):9-12.
- Tondreau T, Lagneaux L, Dejeneffe M, Massy M, Mortier C, Delforge A, Bron D. 2004. Bone marrow-derived mesenchymal stem cells already express specific neural proteins before any differentiation. *Differentiation* 72(7):319-26.
- Tropepe V, Hitoshi S, Sirard C, Mak TW, Rossant J, van der Kooy D. 2001. Direct Neural Fate Specification from Embryonic Stem Cells: A Primitive Mammalian Neural Stem Cell Stage Acquired through a Default Mechanism. *Neuron* 30(1):65-78.
- Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, Tsukamoto AS, Gage FH, Weissman IL. 2000. Direct isolation of human central nervous system stem cells. *PNAS* 97(26):14720-5.
- Vogel G. 2005. CELL BIOLOGY: Ready or Not? Human ES Cells Head Toward the Clinic. *Science* 308(5728):1534-8.
- Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M. 2003. Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* 422(6934):897-901.
- Weimann JM, Johansson CB, Trejo A, Blau HM. 2003. Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant. *Nat Cell Biol* 5(11):959-66.
- Wernig M, Benninger F, Schmandt T, Rade M, Tucker KL, Bussow H, Beck H, Brustle O. 2004. Functional integration of embryonic stem cell-derived neurons in vivo. *J Neurosci* 24(22):5258-68.
- Wichterle H, Lieberam I, Porter JA, Jessell TM. 2002. Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110(3):385-97.
- Wislet-Gendebien S, Hans G, Leprince P, Rigo JM, Moonen G, Rogister B. 2005. Plasticity of Cultured Mesenchymal Stem Cells: Switch from Nestin-Positive to Excitable Neuron-Like Phenotype. *Stem Cells* 23(3):392-402.

- Yan Y, Yang D, Zarnowska ED, Du Z, Werbel B, Valliere C, Pearce RA, Thomson JA, Zhang SC. 2005. Directed Differentiation of Dopaminergic Neuronal Subtypes from Human Embryonic Stem Cells. *Stem Cells* 23(6):781-90.
- Ying QL, Stavridis M, Griffiths D, Li M, Smith A. 2003. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat Biotechnol* 21(2):183-6.
- Yoon Ys, Wecker A, Heyd L, Park JS, Tkebuchava T, Kusano K, Hanley A, Scadova H, Qin G, Cha DH, Johnson KL, Aikawa R, Asahara T, Losordo DW. 2005. Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. *J Clin Invest* 115(2):326-38.
- Zhang J, Li Y, Chen J, Yang M, Katakowski M, Lu M, Chopp M. 2004. Expression of insulin-like growth factor 1 and receptor in ischemic rats treated with human marrow stromal cells. *Brain Research* 1030(1):19-27.
- Zhang SC, Li XJ, Austin JM, Pankratz MT. 2007. Human embryonic stem cells for brain repair? *Philos Trans R Soc Lond B Biol Sci* .
- Zhang SC. 2006. Neural Subtype Specification from Embryonic Stem Cells. *Brain Pathology* 16(2):132-42.
- Zhao LR, Duan WM, Reyes M, Keene CD, Verfaillie CM, Low WC. 2002. Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Exp Neurol* 174(1):11-20.

MANUSCRIPT 2

RAEDT R. VAN DYCKE A., VONCK K., BOON P.

Cell therapy in models for temporal lobe epilepsy.

Seizure. June 11 2007, OnlineEarly Article, doi:[10.1016/j.seizure.2007.05.003](https://doi.org/10.1016/j.seizure.2007.05.003)



REVIEW

Cell therapy in models for temporal lobe epilepsy

R. Raedt ^{*}, A. Van Dycke, K. Vonck, P. Boon

*Laboratory for Clinical and Experimental Neurophysiology, Department of Neurology,
Ghent University Hospital, De Pintelaan 145, B-9000 Ghent, Belgium*

Received 25 January 2007; received in revised form 3 April 2007; accepted 8 May 2007

This manuscript is a comprehensive overview of published studies in which cells are transplanted in a model for TLE. First, a short introduction on TLE and the corresponding animal models is given. Further in the manuscript, two main approaches for cell transplantation in TLE are discussed: cell replacement for reconstruction of damaged networks and the use of cells for local delivery of antiepileptic substances. In the restorative approach, results of transplantation of fetal cells are discussed, followed by the evaluation of neuroprogenitor cell transplantation and the potency of these cells to replace lost brain cells. In the cell-based delivery approach, a distinction is made based on which neuromodulatory substances are secreted by the transplanted cells. Studies using cells which secrete noradrenaline, acetylcholine, GABA and adenosine are critically evaluated.

SUMMARY

For patients with refractory epilepsy it is important to search for alternative treatments. One of these potential treatments could be introducing new cells or modulating endogenous neurogenesis to reconstruct damaged epileptic circuits or to bring neurotransmitter function back into balance. In this review the scientific basis of these cell therapy strategies is discussed and the results are critically evaluated. Research on cell transplantation strategies has mainly been performed in animal models for temporal lobe epilepsy, in which seizure foci or seizure propagation pathways are targeted. Promising results have been obtained, although there remains a lot of debate about the relevance of the animal models, the appropriate target for transplantation, the suitable cell source and the proper time point for transplantation. From the presented studies it should be evident that transplanted cells can survive and sometimes even integrate in an epileptic brain and in a brain that is subjected to epileptogenic interventions. There is evidence that transplanted cells can partially restore damaged structures and/or release substances that modulate existent or induced hyperexcitability. Even though several studies show encouraging results, more studies need to be done in animal models with spontaneous seizures in order to have a better comparison to the human situation.

INTRODUCTION

Epilepsy is characterized by recurrent unprovoked seizures and affects 0.5-1% of the population (Hauser and others, 1991; Brodie and Kwan, 2002). More than 30% of the epilepsy patients have uncontrolled seizures or unacceptable medication-related side effects despite adequate pharmacological treatment (Brodie and Kwan, 2002). These patients have 'refractory epilepsy'. The underlying pathophysiological process that transforms a normal brain into an epileptic brain is termed epileptogenesis.

Epilepsy surgery is an invasive but often curative treatment option that aims at removing the ictal onset zone believed to be responsible for seizure occurrence (Wiebe and others, 2001). For patients in whom the ictal onset zone is not well circumscribed or localized in functional brain tissue, few treatment options are left. The inability to adequately treat all patients with refractory epilepsy provides a continuous impetus to investigate novel forms of treatment.

A possible alternative way of treating refractory patients involves neuromodulation through neurostimulation. In our group, we have demonstrated the efficacy and safety of vagus nerve stimulation (VNS) and deep brain stimulation (DBS) in both patients and experimental animal models for epilepsy (Boon and others, 2001; Vonck and others, 2002; Vonck and others, 2004; Dedeurwaerdere and others, 2005; Dedeurwaerdere and others, 2006). Other possible alternative treatments are newly developed AED (Fisher, 1993), the ketogenic diet (Freeman and others, 1998; Freeman and others, 2006) and transcranial magnetic stimulation (Handforth and others, 1998). In spite of all these developments, a significant number of patients continue to have uncontrolled seizures which makes a further search for alternative treatments mandatory (Jacobs and others, 2001).

A promising treatment option that also receives considerable attention in other neurodegenerative diseases (e.g. Parkinson's disease) is cell therapy (Lindvall and others, 2004; Raedt and Boon, 2005). In general, there are two main strategies that involve the use of cells for the treatment of brain disorders. Firstly, cells can be transplanted to replace lost neurons and/or to release disease modifying substances. Secondly, endogenous cells can be manipulated to affect and modify the disease process.

Temporal lobe epilepsy (TLE) is the most prevalent form of refractory symptomatic epilepsy (Engel, Jr., 2001). Because of its focal nature and the associated cellular defects, this epilepsy syndrome is highly attractive to be treated with cell therapy. This review will highlight the most typical cellular alterations in TLE and then discuss various cell therapy strategies including neural grafting to reconstruct damaged epileptic networks, stimulation of endogenous repair and cell transplantation for local delivery of seizure suppressing substances.

Although the exact cause for the development of seizures in TLE is still under debate, human TLE is very frequently associated with specific pathophysiological changes that are believed to play an important role in the generation or intensification of the epileptic state.

The most frequent lesion in human TLE and status epilepticus (SE) models is hippocampal sclerosis, evident in up to 90% of surgically resected hippocampi (Thom and others, 2002). Hippocampal sclerosis is characterized by extensive gliosis combined with a selective loss of neurons in the dentate gyrus and the hippocampus proper. Neuronal loss involves both glutamatergic neurons (granule cells in dentate gyrus, pyramidal neurons in hippocampus proper) and inhibitory interneurons in dentate hilus and CA1 region. Neuronal cell loss and gliosis can extend to other mesiotemporal regions such as amygdala, entorhinal, perirhinal and temporopolar cortex (Yilmazer-Hanke and others, 2000; Jutila and others, 2001; Salmenpera and others, 2001; Hermann and others, 2002; Wieser, 2004). Most affected neurons are pyramidal neurons of CA1 and CA3 region, excitatory mossy cells in the hilus, and GABAergic inhibitory interneurons also expressing somatostatin, parvalbumin, or neuropeptide Y. Especially the loss of inhibitory interneurons is believed to be a key factor underlying the increased excitability of the epileptic hippocampus (Thompson and others, 1998; Gorter and others, 2001).

Mossy fiber sprouting is the growth of aberrant collaterals of granule cell axons (also called mossy fibers) into the inner molecular layer of the dentate gyrus where they preferentially make synaptic connections with dendrites of other granule neurons, forming excitatory feedback loops (Buckmaster and others, 2002; Scharfman and others, 2003). Mossy fiber sprouting is presumably caused by the loss of normal postsynaptic targets of the granule neurons. One of the arguments for this hypothesis is that the degree of mossy fiber sprouting is correlated to the degree of neuronal loss in hippocampal sclerosis (Cavazos and Cross, 2006). Electrophysiological studies on human hippocampal slices and experimental studies in animal models for TLE have shown that the extend of mossy fiber sprouting is correlated with excitability of the hippocampus (Cavazos and others, 1991; Franck and others, 1995).

By far the most used animal model for TLE, demonstrating spontaneous seizures and typical brain damage, is the status epilepticus (SE) model. In this model SE is evoked by systemic or intracerebral injection of excitotoxins (kainic acid or pilocarpine) or by tetanic electrical stimulation of temporal lobe structures. After a latent period, during which epileptogenesis occurs, spontaneous seizures are displayed (Leite and others, 2002).

Another commonly used model for TLE is the kindling model. In this model temporal lobe structures are repeatedly stimulated by short electrical pulse trains. The animals respond to the stimulation by displaying an electrical discharge on the EEG (afterdischarge) and abnormal behaviour. With increasing number of stimulations rats display more complex afterdischarges or more severe seizures. Rats consistently displaying tonic-clonic seizures are termed fully kindled. Spontaneous seizures are only seen after a large amount of

stimulations but even then gross morphological damage, seen in the status epilepticus model, are not or only moderately evident. Based on the controlled induction of epileptogenesis, the kindling model is a very interesting tool to study the role of events associated with epileptogenesis (McIntyre and others, 2002). Frequently used parameters to assess efficacy of treatments in this model are the current intensity needed to evoke an afterdischarge, termed afterdischarge threshold (ADT), and the number of stimulations needed to fully kindle the rats, termed kindling rate.

STRUCTURAL REPAIR OF DAMAGED EPILEPTIC NETWORKS

CELL TRANSPLANTATION FOR REPAIR OF HIPPOCAMPAL CIRCUITRY

In case of TLE, the sclerotic hippocampus is the most obvious target for circuitry reconstruction given its presumed role in the TLE (Wieser, 2004). However, mediating structural repair of damaged hippocampal networks to restore balance between excitation and inhibition is without doubt an enormous challenge. As already described higher, hippocampal sclerosis involves the loss of different types of both excitatory and inhibitory neurons in different regions throughout the hippocampal structure. Therefore, cellular repair of hippocampal sclerosis will probably require multiple grafts of different cell types throughout the hippocampal structure.

Successful reestablishment of balanced excitatory drive and inhibitory input will demand a great deal of the transplanted cells. They will need to: 1) survive; 2) disperse and/or migrate to appropriate cell layers; 3) generate appropriate phenotypes in correct relative numbers and at the proper locations in the hippocampus; 4) attract suitable afferent input and 5) establish appropriate local and long distance efferent connections with the proper target host and grafted neurons.

The need for proper integration is evident from electrophysiological studies in sclerotic hippocampi which showed that endogenous neurons which display inappropriate synaptic connectivity (Lynch and Sutula, 2000; Buckmaster and others, 2002) and/or integrate at ectopic locations in the hippocampus (Scharfman and others, 2000) are part of hyperexcitable networks. It is therefore very likely that grafted neurons which do not integrate properly could enhance excitability rather than suppressing it.

Indeed, there have been reports which demonstrated pro-epileptic effects of hippocampal transplantation. In a series of experiments, Buzsaki and colleagues transplanted fetal hippocampal tissue chunks or dissociated fetal hippocampal tissue into intact (Buzsaki and others, 1991) or fimbria-fornix lesioned, seizure-prone hippocampus (Buzsaki and others, 1987a; Buzsaki and others, 1987b; Buzsaki and others, 1988a; Buzsaki and others, 1988b; Buzsaki and others, 1989a; Buzsaki and others, 1989c). By performing electrophysiological recordings the authors found that reciprocal electrophysiological connectivity was established between the graft and the intact or lesioned host brain. However, they found that the most typical EEG pattern in the transplant was highly synchronous bursting behaviour with concurrent large amplitude EEG spikes. Spontaneous EEG seizures were also frequently recorded from the graft which spread into the host brain

(Buzsaki and others, 1987a; Buzsaki and others, 1987b). Moreover, spontaneous behavioural convulsions were detected in a high fraction of the transplanted rats (Buzsaki and others, 1988b; Buzsaki and others, 1989a; Buzsaki and others, 1991).

Although the exact mechanism for this transplantation-induced epileptiform activity was not unravelled, the authors suggested that the hyperexcitability of the graft was caused by a lack of afferent control, extensive formation of recurrent excitatory circuitry and insufficient GABAergic inhibition within the graft (Buzsaki and others, 1988b). Buzsaki and colleagues hypothesized that the grafted hippocampal cells served as an epileptic focus that kindled the host brain by repeated seizure induction (Buzsaki and others, 1989a; Buzsaki and others, 1991).

FETAL HIPPOCAMPAL CELL TRANSPLANTATION

In spite of the hurdles, described in the previous chapter, fetal hippocampal neurons have been transplanted into the hippocampus of the intraventricular kainic acid model (Shetty and Turner, 1996; Shetty and Turner, 1997a; Shetty and Turner, 1997b; Shetty and others, 2000; Shetty and Turner, 2000; Zaman and others, 2000; Zaman and Shetty, 2001; Zaman and Shetty, 2003). In this model there is selective loss of CA3 pyramidal neurons (Shetty and Turner, 1996) and subsequent expression of spontaneous limbic seizures (Nadler, 1981; Sater and Nadler, 1988).

A considerable fraction of the transplanted fetal hippocampal cells was able to survive upon transplantation in the damaged CA3 region. However, this survival was severely influenced by postlesion delay (PLD), age of the rats and the type of transplanted cells. Highest survival rates (77%) were seen when fetal CA3 cells were transplanted in young mature rats with a PLD of 4 days (Shetty and Turner, 1996). However if the PLD was longer, the fraction of surviving cells was much lower (e.g. 21 to 31% if PLD was 45 days) (Zaman and others, 2001). Survival in case of transplantation with longer PLD could be dramatically enhanced (up to 99%) by pre-treating the cells with a cocktail of growth factors and an anti-apoptotic factor (Hattiangady and others, 2006). Survival rates of transplanted cells also strongly depended on cell specificity. Survival of CA1 cells, transplanted into the damaged CA3 region, was much lower compared to that of CA3 cells after a PLD of 4 days (respectively 42 % and 77%). When fetal striatal cells were transplanted survival was even worse (only 4 to 12%) (Zaman and others, 2000).

The migration of fetal hippocampal cells upon transplantation in the intraventricular KA model was minimal with the cells remaining clumped at the grafting site (Shetty and others, 1994; Shetty and Turner, 1995). By using retrograde tracing, Shetty and colleagues, showed that grafted fetal hippocampal CA3 neurons formed short-distance efferent projections to ipsilateral CA1 region and entorhinal cortex and long-distance efferent projections to septum and contralateral hippocampus (Shetty and Turner, 1996). However, efferent projections to contralateral hippocampus were only seen in case of homotopic grafting, which means grafting of CA3 neurons in proximity of the damaged CA3 region. If CA3 neurons were transplanted in the CA1 regions or CA1 neurons in the damaged CA3 region no long-distance projections towards contralateral hippocampus were

found (Shetty and Turner, 1997a; Shetty and others, 2000). Growth of host afferent projections into the cluster of grafted cells was also demonstrated. Using histochemical staining and anterograde labelling, afferent cholinergic fibers, mossy fibers and commissural fibers of contralateral CA3 neurons were found in the transplantation area (Shetty and Turner, 1996). Highest density of afferent fibers in the transplant was seen in case of homotopic grafting. The authors hypothesized that the need for homotopic grafting could be due to the fact that axon guidance pathways in the host may be highly specific, requiring accurate placements of the grafts to achieve access (Turner and Shetty, 2003). Both the limited migration and the need for homotopic grafting are very important disadvantages for potential repair of damaged hippocampal circuitry using fetal hippocampal neurons.

Nevertheless, if fetal CA3 neurons were transplanted homotopically, transplantation could result in partial reversal of secondary pathological alterations including aberrant mossy fiber sprouting, possibly by providing an appropriate target (Shetty and Turner, 1997b; Hattiangady and others, 2006). Additionally, loss of glutamic acid decarboxylase (GAD) positive interneurons could be reversed. As the graft did not seem to donate the GAD-positive cells, the authors hypothesized that the loss of CA3 afferents led to a downregulation of GAD protein expression, which was reversed by replacing CA3 cells (Shetty and Turner, 2000).

Unfortunately in their series of experiments the authors did not perform electrophysiological analysis of connectivity, so it remains uncertain whether the transplanted cells also functionally integrated. They also did not monitor for epileptic activity so it is not clear whether grafting of fetal CA3 neurons and the reversal of some pathological secondary changes resulted into a normalization of the imbalance between excitation and inhibition or a dampening of the epileptic activity.

NEURAL STEM/PROGENITOR CELL TRANSPLANTATION

Transplantation of fetal brain tissue has important limitations which will probably always limit its application on a large clinical scale. These limitations include the inability to expand or store fetal cells, resulting in a high number of fetuses needed for one single transplantation (e.g. 6-8 fetal donors to treat one PD patient) (Lindvall, 2001). Another limitation is that purity and viability of the transplant is difficult to control so that outcome of transplantation is highly variable (Bjorklund and others, 2003). Moreover, as already described in the previous chapter, transplanted fetal cells have very limited migratory capabilities and homotopic grafting seems to be required.

Because neural stem/progenitor cells are self-renewing cells which can migrate throughout the brain and are able to generate different neuronal progeny, they could, at least in theory, overcome the limitations of fetal tissue and be a promising alternative cell source. Transplantable neural stem/progenitor cells can be derived in several ways from different sources. They can be produced almost completely *in vitro* starting from embryonic stem cells (ESC) using specific differentiation protocols (Strubing and others, 1995; Okabe and others, 1996; Li and others, 1998; Mujtaba and others, 1999; Carpenter and others, 2001; O'Shea, 2001; Westmoreland and others, 2001; Kim and others, 2002; Wichterle and others, 2002; Conti and others, 2005). Expandable neural

progenitor cells can also be generated by immortalizing neuroepithelial precursor cells, derived from defined embryonic regions, prior to their terminal mitosis. This can be done by transfecting the cells with a vector containing a transcript encoding for a (temperature sensitive) immortalizing oncogene (Martinez-Serrano and Bjorklund, 1997; Whittemore and Onifer, 2000). Neural stem/progenitor cells can also be isolated directly from different regions of the embryonic central nervous system (CNS) but also from restricted areas in the adult brain such as hippocampus, SVZ, striatum, substantia nigra, cortex, spinal cord, septum and optic nerve (Lois and Alvarez-Buylla, 1993; Palmer and others, 1995; Gage and others, 1995; Weiss and others, 1996; Shihabuddin and others, 1997; Palmer and others, 1999; Gage, 2000; Lie and others, 2002).

Following transplantation in the brain, neural stem/progenitor cells seem to be able to functionally integrate into neural networks (Auerbach and others, 2000; Wernig and others, 2004). However, there are only few reports demonstrating the ability of neural stem/progenitor cells to functionally replace lost neurons and reconstruct damaged circuitry (Lindvall and Kokaia, 2006). Transplantation for stroke seems to be most successful with reports on migration of neural progenitor cells towards the lesion with formation of new neurons (Kelly and others, 2004) and reestablishment of neural connections with functional recovery (Ikeda and others, 2005; Hayashi and others, 2006). Systemically injected neural stem cells, in a model for multiple sclerosis, migrate to inflammatory demyelinating lesions, where they can remyelinate axons (Pluchino and others, 2003).

To our knowledge, studies showing structural repair of damaged circuitry in sclerotic hippocampus by transplantation of neural stem/progenitor cells are unavailable. In one study, neural stem cells, isolated from human embryos, have been injected systemically one day after induction of SE with pilocarpine. Transplanted cells were found in the hippocampus, amygdala and pyriform cortex six weeks after transplantation. About 30% of the cells were immunopositive for GABA and parvalbumin, two proteins also expressed by inhibitory interneurons. Surprisingly, the grafted cells did not express the pan-neural markers Neuronal Nuclei (NeuN) or β 3-tub (β -III-tubulin), indicating that they most probably were not neurons. Transplantation did however result in a significant decrease in daily seizure frequency, seizure severity and the number of rats that displayed spontaneous seizures. It was demonstrated that field excitatory postsynaptic potentials (EPSP) in the CA1 region were decreased (Chu and others, 2004). This indicates that transplantation could have anti-epileptogenic effects without evidence of structural repair by the transplanted cells.

A conditionally immortalized neural progenitor cell line, called MHP36, has been developed and has shown potential to replace, at least in part, CA1 pyramidal neurons in models where the CA1 region was specifically damaged, either by excitotoxic lesioning (Virley and others, 1999) or ischemia (Sinden and others, 1997). These MHP36 cells were also transplanted into four sites of the rat brain, extending from the anterior to the posterior pyriform cortex, three weeks after SE. However, in this study no replacement of lost pyramidal CA1 or CA3 neurons was reported but a significant augmentation in the number of seizures was found after transplantation (Meldrum and others, 2000).

In a study, performed at the Ghent University Hospital, adult SVZ-derived neural stem cells were transplanted in the lesioned hippocampus of the intrahippocampal kainic acid SE model (Raedt and others, 2007b). Adult neural stem cells were transplanted 3 days or 3 weeks following lesioning. This resulted in a low (about 1%) but robust survival of the cells for at least six weeks after transplantation. However, only a fraction of the cells differentiated towards neurons while the majority of the cells generated astrocyte-like cells probably contributing to gliosis in response to the lesion.

STIMULATING ENDOGENOUS REPAIR AS A STRATEGY?

In ischemia models neuronal replacement by endogenous neural precursors has been demonstrated in both the striatum and the hippocampus. In the permanent middle cerebral artery occlusion model a small number of lost medium spiny interneurons in the striatum were replaced by endogenous SVZ-derived progenitor cells (Arvidsson and others, 2002; Parent and others, 2002; Yamashita and others, 2006). In the four vessel occlusion model, transient induction of global ischemia leads to selective degeneration of CA1 pyramidal neurons. In this model a fraction of the lost CA1 neurons were replaced by endogenous neural progenitors migrating from the posterior periventricular region (PPV) to the damaged CA1 region (Nakatomi and others, 2002; Bendel and others, 2005). Brief intraventricular infusion of growth factors in the first week after stroke markedly increased reconstitution of CA1 (Nakatomi and others, 2002).

Recently, enhanced proliferation and migration of neural precursor cells from the PPV towards damaged hippocampal CA1 and CA3 regions has been reported in the pilocarpine SE model for TLE. However, these neural precursor cells exclusively generated glial cells in the damaged hippocampal regions without any indication of neuronal replacement (Parent and others, 2006). One strategy that could lead to promoting endogenous repair of sclerotic hippocampus could be to identify the factors which promote neuronal replacement in ischemically damaged hippocampus and/or block neuronal replacement in the sclerotic hippocampus.

In the pilocarpine SE model but also in other models for TLE, seizure-activity stimulates neurogenesis in the granule cell layer of the hippocampus (Parent and others, 1997; Bengzon and others, 1997; Jessberger and others, 2005; Smith and others, 2005). However, as the granule cell layer is relatively spared in case of TLE, it is not yet clear whether this enhanced neurogenesis is an attempt of the brain to repair damage or whether it is a part of the epileptogenic process. It seems that a fraction of the newborn granule cells contributes to the formation of abnormal circuitry by migrating towards ectopic locations in the hippocampus (Scharfman and others, 2000), contributing to mossy fiber sprouting (Parent and others, 1997) or generating a persistent basal dendrite which projects into the hilus and receives synaptic input from sprouted mossy fibers (Shapiro and Ribak, 2006). However, the great majority of the newborn neurons generated in response to seizures, form granule cells which normally integrate into the granule cell layer.

In order to further elaborate on the role of enhanced neurogenesis in response to seizures we recently performed a study, in collaboration with the University of Goteborg, in which we blocked seizure-induced

neurogenesis in rats by using low-dose brain radiation one day before hippocampal kindling. We found that suppression of seizure-induced neurogenesis did not slow down or prevent kindling, indicating that new neurons generated in response to seizures play no major role in kindling epileptogenesis (Raedt and others, 2007a). We believe that further studies are needed in other models to further unravel the role of granule cell neurogenesis in TLE before strategies for suppressing or enhancing endogenous hippocampal neurogenesis could be developed.

CELL GRAFTING FOR LOCAL DELIVERY OF SEIZURE SUPPRESSING SUBSTANCES

Several neurotransmitters and neuromodulators have anticonvulsant effects. Because they are synthesized and secreted by brain-derived cells in normal physiological conditions, they are suitable candidates for cell based delivery therapy. According to this strategy noradrenaline (NA, table 1), acetylcholine (AChE; table 2), GABA (table 3) and adenosine secreting cells (table 4) have been investigated in transplantation studies.

NORADRENALINE SECRETING CELLS (TABLE 1)

The inhibitory effects of NA were reported in temporal lobe epileptogenesis. When extracellular NA levels are artificially augmented by blocking its uptake or by electric stimulation of the noradrenaline rich locus coeruleus (LC), there is a significant attenuation of the kindling rate (McIntyre and others, 1982). On the other hand depletion of the noradrenergic system by injecting 6-hydroxydopamine (6-OHDA) has facilitating effects on kindling (Corcoran, 1988). Transplantation of NA rich fetal LC cells in the hippocampus of NA depleted rats reversed the facilitating effect of the lesion but only when NA release by the graft is under control of the host brain and sufficient in response to kindling stimulation (Barry and others, 1987; Bengzon and others, 1991; Kokaia and others, 1994). Grafting of NA neurons only affected kindling epileptogenesis but not fully kindled seizures. Also no antiepileptic effects could be demonstrated if the LC tissue was transplanted into intact hippocampus (Bengzon and others, 1993). NA-rich neurons have also been transplanted in NA depleted rats into extra-hippocampal regions, such as the amygdala-pyriform cortex. In this experiment grafting only affected seizure development if the transplanted LC neurons re-innervated the host hippocampi bilaterally (Barry and others, 1989). Compared to fetal LC neurons, NA-rich superior cervical ganglion (SCG) neurons demonstrated less survival, integration and NA release (Cenci and others, 1993). Therefore transplantation of SCG neurons had little or no effects on kindling rate in NA-depleted rats (Kokaia and others, 1994). Transplantation of fetal LC neurons in a hippocampus, which is epilepsy prone due to subcortical denervation, gave some protection against picrotoxin-induced behavioural seizures and resulted in less interictal spikes (Buzsaki and others, 1988b).

Fetal LC tissue was also transplanted in the hippocampus of the pilocarpine-induced SE model. After transplantation the number of spontaneous seizures was reduced from approximately 11/week to less than 1/week, with the effect starting between 5 and 6 weeks after grafting surgery and being maximal at about 9 weeks. However, no appropriate control groups were used in this study (Bortolotto and others, 1990). Milder

effects were seen when LC cells were transplanted in immature rats after kainic acid-induced SE. In this study, there was no reduction in the percentage of rats that developed spontaneous seizures, but the transplanted rats displayed fewer spontaneous seizures than sham-transplanted controls. No difference in susceptibility to kindling induced seizures was seen eight months after grafting (Holmes and others, 1991).

We can conclude from these studies that NA-releasing cells can have inhibitory effects on epileptogenesis in specific animal models where the hippocampus is rendered epilepsy prone. NA releasing grafts do not seem to have large effects on established epilepsy. This limits suitability of NA releasing cells for possible clinical applications in TLE.

Table 1 Overview of the transplantation studies with noradrenaline releasing cells

Cell source	Model	Time point of transplantation	Target	Effect on seizures	References
Fetal LC tissue	Kindling after NA-depletion	2 weeks after depletion 6 to 11 months before kindling	Hippocampus	Lower kindling rate	Barry et al. ¹⁰⁶
Fetal LC tissue	Kindling after NA-depletion and intact rats	Fully kindled	Hippocampus	No effect on seizure rate	Bengzon et al. ¹⁰⁸
Fetal LC tissue	Picrotoxin after NA-depletion	10 days after depletion 5 months before picrotoxin injection	Hippocampus	Protection against picrotoxin induced seizures, less interictal spikes	Buszaki et al. ³⁷
Fetal LC tissue	Subcortically denervated epilepsy-prone hippocampus	Chronic epilepsy state	Hippocampus	Reduction in spontaneous seizures	Bortolotto et al. ¹¹¹
Fetal LC tissue	Pilocarpine SE + kindling	2 weeks after SE 6 to 11 months before kindling	Amygdala or pyriform cortex	Decrease of kindling rate	Barry et al. ¹⁰⁹
Fetal LC tissue	KA SE + kindling	10 days after SE 230 days before kindling	Intracerebro-ventricular	Same fraction of epileptic rats; lower seizure frequency; no effect on kindling rate	Holmes et al. ¹¹²
Fetal LC or SCG tissue	Kindling after NA- depletion and aspirative lesion of the fimbria-fornix	4 weeks after depletion 2 weeks after lesion 8 months prekindling	Fimbria-fornix lesion cavity	Lower (LC tissue) or no effect (SCG tissue) on kindling rate	Kokaia et al. ¹⁰⁵

KA: kainic acid; LC: locus coeruleus; TLE: temporal lobe epilepsy; NA: noradrenaline; SCG: superior cervical ganglion; SE: status epilepticus

The septohippocampal system is known to play a role in the regulation of hippocampal excitability. It comprises the medial septum (MS) and the vertical limb of the diagonal band of Broca which are connected with the hippocampus via the fimbria-fornix (FF). These projections are predominantly cholinergic and GABAergic (Rye and others, 1984). Lesioning the FF increases susceptibility for seizure development (Cassel and others, 1987) and produces chronic epileptiform activity like interictal spiking and decreased afterdischarge thresholds (Buzsaki and others, 1989b). These epileptogenic effects are predominantly due to loss of cholinergic afferents, since selective immunolesioning of these afferents with 192 IgG-saporin, induces comparable effects (Kokaia and others, 1996; Ferencz and others, 1997).

Where embryonic, acetylcholine (AChE)-rich basal forebrain tissue was transplanted in the hippocampus of fimbria-fornix lesioned rats, contradictory results were found. Implantation of embryonic basal forebrain tissue into the hippocampus of fimbria-fornix lesioned rats showed that the grafted rats displayed more severe convulsions in response to the proconvulsant pentylenetetrazol (PTZ), but were less reactive to audiogenic stimulation. In this study there was only a poor re-innervation of the deafferented hippocampus (Cassel and others, 1987). In a subsequent study opposite effects were found, with a reduction of the reactivity to PTZ and an increase of the reactivity to sound. In this study the authors reported an improved integration of the graft (Cassel and others, 1991). Due to these conflicting results, conclusions about grafting AChE neurons and seizure susceptibility can not be drawn.

Table 2 Overview of the transplantation studies with acetylcholine releasing cells

Cell source	Model	Time point of transplantation	Target	Effect on seizures	References
Fetal basal forebrain tissue	PTZ and audiogenic stimulation after lesion of the fimbria-fornix	10 days after lesion 1 year before epilepsy induction (PTZ, sound)	Hippocampus	More reactive to PTZ; Less reactive to sound	Cassel et al. ¹¹⁴
Fetal basal forebrain tissue	PTZ and audiogenic stimulation after lesion of the fimbria-fornix	8-9 days after lesion 3, 7 or 12 months before epilepsy induction (PTZ, sound)	Hippocampus	Less reactive to PTZ; More reactive to sound	Cassel et al. ¹¹⁸
Septal-diagonal band tissue	Picrotoxin after 192-IgG-saporin lesioned basal forebrain system	10 days after lesion 5 months before picrotoxin injection	Hippocampus	Decrease in kindling rate	Ferencz et al. ¹¹⁹

PTZ: pentylenetetrazol

Intrahippocampal transplantation of AChE rich fetal septal tissue after saporin-induced lesioning of the forebrain cholinergic system reversed the lesion-induced facilitation of the kindling rate (Ferencz and others, 1998). The authors propose that the suppression of epileptogenesis in the grafted animals may be due to restoration of the cholinergic activation of inhibitory GABAergic interneurons, although there was no direct proof for this assumption.

In all these studies cholinergic grafts were implanted before induction of epileptogenesis. Whether or not grafts of cholinergic-rich neurons can have anticonvulsive effects after the epileptic syndrome has been established is still unclear.

GABA RELEASING CELLS (TABLE 3)

Initial evidence that administration of GABA inhibits seizure activity was reported in studies using neurotransmitter application in dogs (Meldrum, 1978). Peripheral administration of GABA or GABA agonists has several limitations, such as limited capacity to cross the blood-brain barrier, undesirable side effects and proconvulsant activity in primates and humans (Meldrum, 1978), due to the diffuse stimulation of GABA_A receptors within the brain. An alternative to increase GABA release at the epileptic focus is to implant GABA-releasing cells.

In different regions of the brain, contributing to the manifestation of seizures, a consistent loss of glutamic acid decarboxylase (GAD)-positive interneurons has been demonstrated. These regions are the substantia nigra pars reticulata (SNr) (Loscher and Schwark, 1985; Turski and others, 1989), the basolateral amygdala (BLA) (Lehmann and others, 1998), the striatum (Loscher and Schwark, 1987), the pyriform cortex (PC) (Lehmann and others, 1998), and the hippocampus (Shetty and Turner, 2000; Gorter and others, 2001). GABA releasing cells have been grafted in some of these structures in different models for TLE.

GABA-rich fetal striatal tissue has been transplanted into the SNr of fully amygdala kindled rats (Loscher and others, 1998). SNr was chosen because of its presumed role in the spreading of seizure activity (Loscher and Ebert, 1996). After transplantation, a significant increase in ADT was seen. Also a significant decrease in seizure severity was evident. These seizure-suppressing effects were transient and disappeared in the weeks following transplantation.

As an alternative for fetal GABAergic cells, Thompson and coworkers have engineered conditionally immortalized mouse neurons to deliver GABA by driving GAD₆₅ expression that could be shut down by the administration of doxycycline. This cell line has been transplanted into the SNr (Thompson and others, 2000), the pyriform cortex (Gernert and others, 2002) and the hippocampal dentate gyrus (Thompson, 2005) of rats prior to kindling. The effect of transplantation in the SNr was dependent on the location within the SNr. Transplantation in the posterior SNr significantly facilitated kindling development. When cells were transplanted in the anterior SNr kindling rate decreased but not significantly (Thompson and others, 2000). Transplantation of the cells in the pyriform cortex caused a temporary increase in ADT but did not effect

kindling rate (Gernert and others, 2002). The reason why this anticonvulsant effect was partial and transient could have several explanations such as a decrease of *in vivo* GABA-release, progressive cell death of transplanted cells or down regulation of GABA receptors in the host tissue. Transplantation of the cells in the hippocampus improved the results. After transplantation an elevation of ADT, a slower entorhinal kindling rate and a longer latency between entorhinal stimulation and behavioural seizures was found. The transplanted cells showed limited survival and were detected only in 30% of the transplanted animals three weeks after grafting (Thompson, 2005). These GABA releasing cells have also been transplanted into the anterior substantia nigra 45-65 days after pilocarpine induced SE (Thompson and Suchomelova, 2004). Seven to ten days following transplantation there was a robust suppression of behavioural seizures and a reduction in interictal spikes. The evaluation of the seizure suppressing effect of GABA releasing transplants ended 13 days after transplantation, while it would have been interesting to investigate whether this anticonvulsant effect was long lasting.

The effect of GABA releasing cells on seizures are more convincing than earlier studies with NA and AchE releasing cells. Long-term cell survival and video-EEG monitoring is required in animal models with spontaneous seizures in order to evaluate the duration of the anticonvulsant effect and the possible presence of side effects.

Table 3 Overview of the transplantation studies with GABA releasing cells

Cell source	Model	Time point of transplantation	Target	Effect on seizures	References
Fetal striatal tissue	Amygdala kindling	Fully kindled	SN	Transient higher ADT Less severe seizures	Löscher et al. ¹²⁵
GABA secreting cortical neural cell line	Entorhinal cortex kindling	10 days before kindling	SN	Posterior SN: higher kindling rate Anterior SN: lower kindling rate	Thompson et al. ¹²⁷
GABA secreting cortical neural cell line	Entorhinal cortex kindling	12 days before kindling	Pyriform cortex	Increase in ADT; No difference in kindling rate	Gernert et al. ¹²⁸
GABA secreting cortical neural cell line	Entorhinal cortex kindling	7- 10 days before kindling	Hippocampus	Higher ADT Lower ADD Lower kindling rate	Thompson et al. ¹²⁹
GABA secreting cortical neural cell line	Pilocarpine SE	45-65 days after SE	Anterior SN	Seizure-suppression up to 13 days after transplantation	Thompson et al. ¹³⁰

ADD: afterdischarge duration; ADT: afterdischarge threshold; SE: status epilepticus; SN: substantia nigra

ADENOSINE RELEASING CELLS (TABLE 4)

Adenosine and its analogues have powerful antiseizure and neuroprotective activities (Lee and others, 1984; Fredholm, 1997). During epileptic seizures or status epilepticus, extracellular adenosine concentrations are elevated. This is considered to be an endogenous protective mechanism in order to control the ongoing seizure. Unfortunately, during the process of epileptogenesis the tonic inhibition of adenosine decreases due to downregulation of its A1-receptors and increased break down of adenosine by adenosine kinase (Boison, 2005; Boison, 2006). However, because of its great potential to control seizures, adenosine might be a good alternative substance for treating epilepsy.

When administered systemically, adenosine and adenosine agonists cause adverse effects which have prevented its therapeutic use (Gouder and others, 2003). Therefore experiments have been set up in which adenosine was released locally in the brain of kindled rats by synthetic polymers (Boison and others, 1999). Analysis of adenosine release revealed that a release of 20-50 ng/day by the polymer is sufficient to provide protection against seizures. The anticonvulsant effects lasted up to fourteen days after transplantation of the polymer. At this moment adenosine release was reduced to less than 10 ng per day. These experiments showed that the amounts of adenosine required to locally suppress seizure activity were in the range, achievable for adenosine released from cell sources. Transplantation of cells, which have the capacity to survive and permanently release adenosine, is a promising tool to achieve a more sustained suppression of seizure activity. Baby hamster kidney fibroblasts (Huber and others, 2001), mouse myoblasts (Guttinger and others, 2005b) and mouse ESC -derived glia (Fedele and others, 2004; Guttinger and others, 2005a), all genetically engineered to release adenosine, have been transplanted in fully kindled rats. Transplantation of engineered hamster kidney fibroblasts and mouse myoblasts resulted in an almost complete suppression of kindled seizures up to 14 days after transplantation. After 14 days there was a gradual loss of seizure protection which could be attributed to a limited survival of the cells (Huber and others, 2001; Guttinger and others, 2005b). This survival dependant effect was even more evident from the experiments where mouse embryonic stem cell-derived glial cells were transplanted into fully kindled rats. Three days after transplantation complete suppression of seizures in 100 % of the animals and 90% cell viability was found. Seven days after transplantation, seizure suppression was lost and viable cells were no longer detectable.

In all studies seizure-suppressant effects could be contributed to the adenosine release since injection of the A1 receptor antagonist DPCPX (8-cyclopentyl-1,3-dipropyl-xanthine) abolished the adenosine-induced seizure suppressing effects. From these studies it is evident that the search for a cell source, which is able to survive for prolonged time in the brain while continuously secreting anti-seizure substances, is of major importance to develop cell therapies for the treatment of refractory TLE patients.

Table 4 Overview of the transplantation studies with adenosine releasing cells

Cell source	Model	Time point of transplantation	Target	Effect on seizures	References
Encapsulated, engineered baby hamster kidney cells	Hippocampal kindling	Fully kindled	Lateral ventricle	Day 1-14: almost complete suppression of seizures Day 14-24: gradual loss of seizure protection	Huber et al. ¹³⁷
Encapsulated, engineered adult mouse myoblasts	Hippocampal kindling	Fully kindled	Lateral ventricle	Day 1-7: complete suppression of seizures in all rats Day 7-week 8: gradual loss of seizures protection	Güttinger et al. ¹³⁸
Encapsulated, engineered embryonic stem cells derived glia	Hippocampal kindling	Fully kindled	Lateral ventricle	Day 3: complete suppression of seizures in all rats Day 7: no seizure suppression	Güttinger et al. ¹⁴⁰

CONCLUSION

In TLE, structural changes in the hippocampus are believed to play a key role in the generation of epileptic seizures. However, given the complexity of hippocampal circuitry and cell damage in case of hippocampal sclerosis, structural repair of epileptic hippocampal networks will require complex transplantation strategies in which proper integration and rewiring of the implanted neurons will be of crucial importance. Fetal hippocampal transplantation has been successful in reversing some pathological changes but important disadvantages, such as limited migration and the need for homotopic transplantation, have urged the search for alternative cell types. Exogenous and endogenous neural progenitor cells could be used for the repair hippocampal damage. However, increased knowledge about injury-induced neurogenesis and differentiation pathways will be necessary in order to guide the cells towards the cell type they have to replace and prevent them from contributing to pathological processes such as gliosis.

In another strategy, cells are transplanted for the release of neurotransmitters or neuromodulatory agents. Transplantation studies for epilepsy have mainly grafted therapeutic cells before the epileptogenesis induction. Although there are already promising results with certain substances, such as GABA and adenosine, further *in vivo* studies in animal models with spontaneous seizures are mandatory to initiate extrapolations to the human situation.

REFERENCES

- Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. 2002. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med* 8(9):963-70.
- Auerbach JM, Eiden MV, McKay RD. 2000. Transplanted CNS stem cells form functional synapses in vivo. *European Journal of Neuroscience* 12(5):1696-704.
- Barry DI, Kikvadze I, Brundin P, Bolwig TG, Bjorklund A, Lindvall O. 1987. Grafted noradrenergic neurons suppress seizure development in kindling-induced epilepsy. *Proc Natl Acad Sci U S A* 84(23):8712-5.
- Barry DI, Wanscher B, Kragh J, Bolwig TG, Kokaia M, Brundin P, Bjorklund A, Lindvall O. 1989. Grafts of fetal locus coeruleus neurons in rat amygdala-piriform cortex suppress seizure development in hippocampal kindling. *Exp Neurol* 106(2):125-32.
- Bendel O, Bueters T, von EM, Ove OS, Sandin J, von EG. 2005. Reappearance of hippocampal CA1 neurons after ischemia is associated with recovery of learning and memory. *J Cereb Blood Flow Metab* 25(12):1586-95.
- Bengzon J, Brundin P, Kalen P, Kokaia M, Lindvall O. 1991. Host regulation of noradrenaline release from grafts of seizure-suppressant locus coeruleus neurons. *Exp Neurol* 111(1):49-54.
- Bengzon J, Kokaia Z, Elmer E, Nanobashvili A, Kokaia M, Lindvall O. 1997. Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. *Proc Natl Acad Sci U S A* 94(19):10432-7.
- Bengzon J, Kokaia Z, Lindvall O. 1993. Specific functions of grafted locus coeruleus neurons in the kindling model of epilepsy. *Exp Neurol* 122(1):143-54.
- Bjorklund A, Dunnett SB, Brundin P, Stoessl AJ, Freed CR, Breeze RE, Levivier M, Peschanski M, Studer L, Barker R. 2003. Neural transplantation for the treatment of Parkinson's disease. *Lancet Neurol* 2(7):437-45.
- Boison D. 2005. Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. *Neuroscientist* 11(1):25-36.
- Boison D. 2006. Adenosine kinase, epilepsy and stroke: mechanisms and therapies. *Trends Pharmacol Sci* 27(12):652-8.
- Boison D, Scheurer L, Tseng JL, Aebischer P, Mohler H. 1999. Seizure suppression in kindled rats by intraventricular grafting of an adenosine releasing synthetic polymer. *Exp Neurol* 160(1):164-74.
- Boon P, Vonck K, De Reuck J, Caemaert J. 2001. Vagus nerve stimulation for refractory epilepsy. *Seizure-European Journal of Epilepsy* 10(6):448-55.
- Bortolotto ZA, Calderazzo L, Cavalheiro EA. 1990. Some evidence that intrahippocampal grafting of noradrenergic neurons suppresses spontaneous seizures in epileptic rats. *Braz J Med Biol Res* 23(12):1267-9.
- Brodie MJ, Kwan P. 2002. Staged approach to epilepsy management. *Neurology* 58(8 Suppl 5):S2-S8.
- Buckmaster PS, Zhang GF, Yamawaki R. 2002. Axon sprouting in a model of temporal lobe epilepsy creates a predominantly excitatory feedback circuit. *J Neurosci* 22(15):6650-8.
- Buzsaki G, Bayardo F, Miles R, Wong RK, Gage FH. 1989a. The grafted hippocampus: an epileptic focus. *Exp Neurol* 105(1):10-22.
- Buzsaki G, Czopf J, Kondakor I, Bjorklund A, Gage FH. 1987a. Cellular activity of intracerebrally transplanted fetal hippocampus during behavior. *Neuroscience* 22(3):871-83.
- Buzsaki G, Freund T, Bjorklund A, Gage FH. 1988a. Restoration and deterioration of function by brain grafts in the septohippocampal system. *Prog Brain Res* 78:69-77.
- Buzsaki G, Gage FH, Kellenyi L, Bjorklund A. 1987b. Behavioral dependence of the electrical activity of intracerebrally transplanted fetal hippocampus. *Brain Res* 400(2):321-33.
- Buzsaki G, Masliah E, Chen LS, Horvath Z, Terry R, Gage FH. 1991. Hippocampal grafts into the intact brain induce epileptic patterns. *Brain Res* 554(1-2):30-7.
- Buzsaki G, Ponomareff G, Bayardo F, Shaw T, Gage FH. 1988b. Suppression and induction of epileptic activity by neuronal grafts. *Proc Natl Acad Sci U S A* 85(23):9327-30.

- Buzsaki G, Ponomareff GL, Bayardo F, Ruiz R, Gage FH. 1989b. Neuronal activity in the subcortically denervated hippocampus: a chronic model for epilepsy. *Neuroscience* 28(3):527-38.
- Buzsaki G, Wiesner J, Henriksen SJ, Gage FH. 1989c. Long-term potentiation of evoked and spontaneous neuronal activity in the grafted hippocampus. *Exp Brain Res* 76(2):401-8.
- Carpenter MK, Inokuma MS, Denham J, Mujtaba T, Chiu CP, Rao MS. 2001. Enrichment of neurons and neural precursors from human embryonic stem cells. *Exp Neurol* 172(2):383-97.
- Cassel JC, Kelche C, Will BE. 1987. Susceptibility to pentylenetetrazol-induced and audiogenic seizures in rats with selective fimbria-fornix lesions and intrahippocampal septal grafts. *Exp Neurol* 97(3):564-76.
- Cassel JC, Kelche C, Will BE. 1991. Susceptibility to pentylenetetrazol-induced and audiogenic-seizures in rats given aspirative lesions of the fimbria-fornix pathways followed by intrahippocampal grafts-a time course approach. *Restorative Neurology and Neuroscience* 3(2):55-64.
- Cavazos JE, Golarai G, Sutula TP. 1991. Mossy fiber synaptic reorganization induced by kindling: time course of development, progression, and permanence. *J Neurosci* 11(9):2795-803.
- Cavazos JE, Cross DJ. 2006. The role of synaptic reorganization in mesial temporal lobe epilepsy. *Epilepsy & Behavior* 8(3):483-93.
- Cenci MA, Nilsson OG, Kalen P, Bjorklund A. 1993. Characterization of in vivo noradrenaline release from superior cervical ganglia or fetal locus coeruleus transplanted to the subcortically deafferented hippocampus in the rat. *Exp Neurol* 122(1):73-87.
- Chu K, Kim M, Jung KH, Jeon D, Lee ST, Kim J, Jeong SW, Kim SU, Lee SK, Shin HS, Roh JK. 2004. Human neural stem cell transplantation reduces spontaneous recurrent seizures following pilocarpine-induced status epilepticus in adult rats. *Brain Res* 1023(2):213-21.
- Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying QL, Cattaneo E, Smith A. 2005. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol* 3(9):e283.
- Corcoran ME. 1988. Characteristics of accelerated kindling after depletion of noradrenaline in adult rats. *Neuropharmacology* 27(10):1081-4.
- Dedeurwaerdere S, Gilby K, Vonck K, Delbeke J, Boon P, McIntyre D. 2006. Vagus nerve stimulation does not affect spatial memory in fast rats, but has both anti-convulsive and pro-convulsive effects on amygdala-kindled seizures. *Neuroscience* 140(4):1443-51.
- Dedeurwaerdere S, Vonck K, Van Hese P, Wadman W, Boon P. 2005. The acute and chronic effect of vagus nerve stimulation in genetic absence epilepsy rats from Strasbourg (GAERS). *Epilepsia* 46:94-7.
- Engel J, Jr. 2001. Mesial Temporal Lobe Epilepsy: What Have We Learned? *Neuroscientist* 7(4):340-52.
- Fedele DE, Koch P, Scheurer L, Simpson EM, Mohler H, Brustle O, Boison D. 2004. Engineering embryonic stem cell derived glia for adenosine delivery. *Neurosci Lett* 370(2-3):160-5.
- Ferencz I, Kokaia M, Elmer E, Keep M, Kokaia Z, Lindvall O. 1998. Suppression of kindling epileptogenesis in rats by intrahippocampal cholinergic grafts. *European Journal of Neuroscience* 10(1):213-20.
- Ferencz I, Kokaia M, Keep M, Elmer E, Metsis M, Kokaia Z, Lindvall O. 1997. Effects of cholinergic denervation on seizure development and neurotrophin messenger RNA regulation in rapid hippocampal kindling. *Neuroscience* 80(2):389-99.
- Fisher RS. 1993. Emerging antiepileptic drugs. *Neurology* 43(11 Suppl 5):S12-S20.
- Franck JE, Pokorny J, Kunkel DD, Schwartzkroin PA. 1995. Physiologic and morphologic characteristics of granule cell circuitry in human epileptic hippocampus. *Epilepsia* 36(6):543-58.
- Fredholm BB. 1997. Adenosine and neuroprotection. *Int Rev Neurobiol* 40:259-80.
- Freeman J, Veggiotti P, Lanzi G, Tagliabue A, Perucca E. 2006. The ketogenic diet: from molecular mechanisms to clinical effects. *Epilepsy Res* 68(2):145-80.
- Freeman JM, Vining EP, Pillas DJ, Pyzik PL, Casey JC, Kelly L, and Millicent. 1998. The Efficacy of the Ketogenic Diet---1998: A Prospective Evaluation of Intervention in 150áChildren. *Pediatrics* 102(6):1358-63.

Gage FH. 2000. Mammalian neural stem cells. *Science* 287(5457):1433-8.

Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, Peterson DA, Suhr ST, Ray J. 1995. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci U S A* 92(25):11879-83.

Gernert M, Thompson KW, Loscher W, Tobin AJ. 2002. Genetically engineered GABA-producing cells demonstrate anticonvulsant effects and long-term transgene expression when transplanted into the central piriform cortex of rats. *Exp Neurol* 176(1):183-92.

Gorter JA, van Vliet EA, Aronica E, Lopes da Silva FH. 2001. Progression of spontaneous seizures after status epilepticus is associated with mossy fibre sprouting and extensive bilateral loss of hilar parvalbumin and somatostatin-immunoreactive neurons. *European Journal of Neuroscience* 13(4):657-69.

Gouder N, Fritschy JM, Boison D. 2003. Seizure suppression by adenosine A1 receptor activation in a mouse model of pharmacoresistant epilepsy. *Epilepsia* 44(7):877-85.

Guttinger M, Fedele D, Koch P, Padrun V, Pralong WF, Brustle O, Boison D. 2005a. Suppression of kindled seizures by paracrine adenosine release from stem cell-derived brain implants. *Epilepsia* 46(8):1162-9.

Guttinger M, Padrun V, Pralong WF, Boison D. 2005b. Seizure suppression and lack of adenosine A1 receptor desensitization after focal long-term delivery of adenosine by encapsulated myoblasts. *Exp Neurol* 193(1):53-64.

Handforth A, DeGiorgio CM, Schachter SC, Uthman BM, Naritoku DK, Tecoma ES, Henry TR, Collins SD, Vaughn BV, Gilmartin RC, Labar DR, Morris GL, III, Salinsky MC, Osorio I, Ristanovic RK, Labiner DM, Jones JC, Murphy JV, Ney GC, Wheless JW. 1998. Vagus nerve stimulation therapy for partial-onset seizures: a randomized active-control trial. *Neurology* 51(1):48-55.

Hattiangady B, Rao MS, Zaman V, Shetty AK. 2006. Incorporation of embryonic CA3 cell grafts into the adult hippocampus at 4-months after injury: Effects of combined neurotrophic supplementation and caspase inhibition. *Neuroscience* 139(4):1369-83.

Hauser WA, Annegers JF, Kurland LT. 1991. Prevalence of epilepsy in Rochester, Minnesota: 1940-1980. *Epilepsia* 32(4):429-45.

Hayashi J, Takagi Y, Fukuda H, Imazato T, Nishimura M, Fujimoto M, Takahashi J, Hashimoto N, Nozaki K. 2006. Primate embryonic stem cell-derived neuronal progenitors transplanted into ischemic brain. *J Cereb Blood Flow Metab* 26(7):906-14.

Hermann B, Seidenberg M, Bell B, Rutecki P, Sheth R, Ruggles K, Wendt G, O'Leary D, Magnotta V. 2002. The neurodevelopmental impact of childhood-onset temporal lobe epilepsy on brain structure and function. *Epilepsia* 43(9):1062-71.

Holmes GL, Thompson JL, Huh K, Holmes C, Carl GF. 1991. Effect of neural transplants on seizure frequency and kindling in immature rats following kainic acid. *Brain Res Dev Brain Res* 64(1-2):47-56.

Huber A, Padrun V, Deglon N, Aebischer P, Mohler H, Boison D. 2001. Grafts of adenosine-releasing cells suppress seizures in kindling epilepsy. *Proc Natl Acad Sci U S A* 98(13):7611-6.

Ikeda R, Kurokawa MS, Chiba S, Yoshikawa H, Ide M, Tadokoro M, Nito S, Nakatsuji N, Kondoh Y, Nagata K, Hashimoto T, Suzuki N. 2005. Transplantation of neural cells derived from retinoic acid-treated cynomolgus monkey embryonic stem cells successfully improved motor function of hemiplegic mice with experimental brain injury. *Neurobiology of Disease* 20(1):38-48.

Jacobs MP, Fischbach GD, Davis MR, Dichter MA, Dingledine R, Lowenstein DH, Morrell MJ, Noebels JL, Rogawski MA, Spencer SS, Theodore WH. 2001. Future directions for epilepsy research. *Neurology* 57(9):1536-42.

Jessberger S, Romer B, Babu H, Kempermann G. 2005. Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. *Exp Neurol* 196(2):342-51.

Jutila L, Ylinen A, Partanen K, Alafuzoff I, Mervaala E, Partanen J, Vapalahti M, Vainio P, Pitkanen A. 2001. MR volumetry of the entorhinal, perirhinal, and temporopolar cortices in drug-refractory temporal lobe epilepsy. *American Journal of Neuroradiology* 22(8):1490-501.

- Kelly S, Bliss TM, Shah AK, Sun GH, Ma M, Foo WC, Masel J, Yenari MA, Weissman IL, Uchida N, Palmer T, Steinberg GK. 2004. Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. *PNAS* 101(32):11839-44.
- Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-Pernaute R, Bankiewicz K, McKay R. 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418(6893):50-6.
- Kokaia M, Cenci MA, Elmer E, Nilsson OG, Kokaia Z, Bengzon J, Bjorklund A, Lindvall O. 1994. Seizure development and noradrenaline release in kindling epilepsy after noradrenergic reinnervation of the subcortically deafferented hippocampus by superior cervical ganglion or fetal locus coeruleus grafts. *Exp Neurol* 130(2):351-61.
- Kokaia M, Ferencz I, Leanza G, Elmer E, Metsis M, Kokaia Z, Wiley RG, Lindvall O. 1996. Immunolesioning of basal forebrain cholinergic neurons facilitates hippocampal kindling and perturbs neurotrophin messenger RNA regulation. *Neuroscience* 70(2):313-27.
- Lee KS, Schubert P, Heinemann U. 1984. The anticonvulsive action of adenosine: a postsynaptic, dendritic action by a possible endogenous anticonvulsant. *Brain Res* 321(1):160-4.
- Lehmann H, Ebert U, Loscher W. 1998. Amygdala-kindling induces a lasting reduction of GABA-immunoreactive neurons in a discrete area of the ipsilateral piriform cortex. *Synapse* 29(4):299-309.
- Leite JP, Garcia-Cairasco N, Cavalheiro EA. 2002. New insights from the use of pilocarpine and kainate models. *Epilepsy Res* 50(1-2):93-103.
- Li M, Pevny L, Lovell-Badge R, Smith A. 1998. Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr Biol* 8(17):971-4.
- Lie DC, Dziezwczapolski G, Willhoite AR, Kaspar BK, Shults CW, Gage FH. 2002. The adult substantia nigra contains progenitor cells with neurogenic potential. *J Neurosci* 22(15):6639-49.
- Lindvall O. 2001. Parkinson disease. Stem cell transplantation. *Lancet* 358 Suppl:S48.
- Lindvall O, Kokaia Z. 2006. Stem cells for the treatment of neurological disorders. *Nature* 441(7097):1094-6.
- Lindvall O, Kokaia Z, Martinez-Serrano A. 2004. Stem cell therapy for human neurodegenerative disorders-how to make it work. *Nat Med* 10 Suppl:S42-S50.
- Lois C, Alvarez-Buylla A. 1993. Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc Natl Acad Sci U S A* 90(5):2074-7.
- Loscher W, Ebert U. 1996. Basic mechanisms of seizure propagation: targets for rational drug design and rational polypharmacy. *Epilepsy Res Suppl* 11:17-43.:17-43.
- Loscher W, Ebert U, Lehmann H, Rosenthal C, Nikkhah G. 1998. Seizure suppression in kindling epilepsy by grafts of fetal GABAergic neurons in rat substantia nigra. *J Neurosci Res* 51(2):196-209.
- Loscher W, Schwark WS. 1985. Evidence for impaired GABAergic activity in the substantia nigra of amygdaloid kindled rats. *Brain Res* 339(1):146-50.
- Loscher W, Schwark WS. 1987. Further evidence for abnormal GABAergic circuits in amygdala-kindled rats. *Brain Res* 420(2):385-90.
- Lynch M, Sutula T. 2000. Recurrent excitatory connectivity in the dentate gyrus of kindled and kainic acid-treated rats. *J Neurophysiol* 83(2):693-704.
- Martinez-Serrano A, Bjorklund A. 1997. Immortalized neural progenitor cells for CNS gene transfer and repair. *Trends Neurosci* 20(11):530-8.
- McIntyre DC, Edson N, Chao G, Knowles V. 1982. Differential effect of acute vs chronic desmethylinipramine on the rate of amygdala kindling in rats. *Exp Neurol* 78(1):158-66.
- McIntyre DC, Poulter MO, Gilby K. 2002. Kindling: some old and some new. *Epilepsy Research* 50(1-2):79-92.
- Meldrum BS. 1978. Gamma-aminobutyric acid and the search for new anticonvulsant drugs. *Lancet* 2(8084):304-6.

- Meldrum BS, Chapman AG, Tang E, Keaney K, Patel S, Chadwick A, Hodges H. 2000. Cell grafts in epilepsy: therapeutic prospects and problems. *Acta Neurologica Scandinavica* 102:46-7.
- Mujtaba T, Piper DR, Kalyani A, Groves AK, Lucero MT, Rao MS. 1999. Lineage-restricted neural precursors can be isolated from both the mouse neural tube and cultured ES cells. *Dev Biol* 214(1):113-27.
- Nadler JV. 1981. Minireview. Kainic acid as a tool for the study of temporal lobe epilepsy. *Life Sci* 29(20):2031-42.
- Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M. 2002. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* 110(4):429-41.
- O'Shea KS. 2001. Neuronal differentiation of mouse embryonic stem cells: lineage selection and forced differentiation paradigms. *Blood Cells Mol Dis* 27(3):705-12.
- Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD. 1996. Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. *Mech Dev* 59(1):89-102.
- Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH. 1999. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J Neurosci* 19(19):8487-97.
- Palmer TD, Ray J, Gage FH. 1995. FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Mol Cell Neurosci* 6(5):474-86.
- Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM. 2002. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol* 52(6):802-13.
- Parent JM, von dem BN, Lowenstein DH. 2006. Prolonged seizures recruit caudal subventricular zone glial progenitors into the injured hippocampus. *Hippocampus* 16(3):321-8.
- Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH. 1997. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci* 17(10):3727-38.
- Pluchino S, Quattrini A, Brambilla E, Gritti A, Salani G, Dina G, Galli R, Del Carro U, Amadio S, Bergami A, Furlan R, Comi G, Vescovi AL, Martino G. 2003. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* 422(6933):688-94.
- Raedt R, Boon P. 2005. Cell therapy for neurological disorders: a comprehensive review. *Acta Neurol Belg* 105(3):158-70.
- Raedt R, Boon P, Persson A, Allborn AM, Boterberg T, Van Dycke A, Linder B, De Smedt T, Wadman WJ, Ben-Menachem E, Eriksson PS. 2007a. Radiation of the rat brain suppresses seizure-induced neurogenesis and transiently enhances excitability during kindling acquisition. *Epilepsia* submitted.
- Raedt R, Van Dycke A, Waeytens A, Van de Kerckhove B, Vonck K, Wadman W, Boon P. 2007b. Neural precursor cells demonstrate long term survival and differentiate mainly towards astrocytes upon transplantation in sclerotic hippocampus. *Exp Neurol* submitted.
- Rye DB, Wainer BH, Mesulam MM, Mufson EJ, Saper CB. 1984. Cortical projections arising from the basal forebrain: a study of cholinergic and noncholinergic components employing combined retrograde tracing and immunohistochemical localization of choline acetyltransferase. *Neuroscience* 13(3):627-43.
- Salmenpera T, Kalviainen R, Partanen K, Pitkanen A. 2001. Hippocampal and amygdaloid damage in partial epilepsy - A cross-sectional MRI study of 241 patients. *Epilepsy Research* 46(1):69-82.
- Sater RA, Nadler JV. 1988. On the relation between seizures and brain lesions after intracerebroventricular kainic acid. *Neuroscience Letters* 84(1):73-8.
- Scharfman HE, Goodman JH, Sollas AL. 2000. Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. *J Neurosci* 20(16):6144-58.

- Scharfman HE, Sollas AL, Berger RE, Goodman JH. 2003. Electrophysiological evidence of monosynaptic excitatory transmission between granule cells after seizure-induced mossy fiber sprouting. *J Neurophysiol* 90(4):2536-47.
- Shapiro LA, Ribak CE. 2006. Newly born dentate granule neurons after pilocarpine-induced epilepsy have hilar basal dendrites with immature synapses. *Epilepsy Res* 69(1):53-66.
- Shetty AK, Madison RD, Bradley J, Turner DA. 1994. Quantitative graft integration of fetal hippocampal transplants labeled with 5' bromodeoxyuridine into normal adult hippocampus. *Exp Neurol* 126(2):205-24.
- Shetty AK, Turner DA. 1995. Enhanced cell survival in fetal hippocampal suspension transplants grafted to adult rat hippocampus following kainate lesions: a three-dimensional graft reconstruction study. *Neuroscience* 67(3):561-82.
- Shetty AK, Turner DA. 1996. Development of fetal hippocampal grafts in intact and lesioned hippocampus. *Prog Neurobiol* 50(5-6):597-653.
- Shetty AK, Turner DA. 1997a. Development of long-distance efferent projections from fetal hippocampal grafts depends upon pathway specificity and graft location in kainate-lesioned adult hippocampus. *Neuroscience* 76(4):1205-19.
- Shetty AK, Turner DA. 1997b. Fetal hippocampal cells grafted to kainate-lesioned CA3 region of adult hippocampus suppress aberrant supragranular sprouting of host mossy fibers. *Exp Neurol* 143(2):231-45.
- Shetty AK, Turner DA. 2000. Fetal hippocampal grafts containing CA3 cells restore host hippocampal glutamate decarboxylase-positive interneuron numbers in a rat model of temporal lobe epilepsy. *J Neurosci* 20(23):8788-801.
- Shetty AK, Zaman V, Turner DA. 2000. Pattern of long-distance projections from fetal hippocampal field CA3 and CA1 cell grafts in lesioned CA3 of adult hippocampus follows intrinsic character of respective donor cells. *Neuroscience* 99(2):243-55.
- Shihabuddin LS, Ray J, Gage FH. 1997. FGF-2 is sufficient to isolate progenitors found in the adult mammalian spinal cord. *Exp Neurol* 148(2):577-86.
- Sinden JD, Rashid-Doubell F, Kershaw TR, Nelson A, Chadwick A, Jat PS, Noble MD, Hodges H, Gray JA. 1997. Recovery of spatial learning by grafts of a conditionally immortalized hippocampal neuroepithelial cell line into the ischaemia-lesioned hippocampus. *Neuroscience* 81(3):599-608.
- Smith PD, Mclean KJ, Murphy MA, Turnley AM, Cook MJ. 2005. Seizures, not hippocampal neuronal death, provoke neurogenesis in a mouse rapid electrical amygdala kindling model of seizures. *Neuroscience* 136(2):405-15.
- Strubing C, Ahnert-Hilger G, Shan J, Wiedenmann B, Hescheler J, Wobus AM. 1995. Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. *Mech Dev* 53(2):275-87.
- Thom M, Sisodiya SM, Beckett A, Martinian L, Lin WR, Harkness W, Mitchell TN, Craig J, Duncan J, Scaravilli F. 2002. Cytoarchitectural abnormalities in hippocampal sclerosis. *Journal of Neuropathology and Experimental Neurology* 61(6):510-9.
- Thompson K, Anantharam V, Behrstock S, Bongarzone E, Campagnoni A, Tobin AJ. 2000. Conditionally immortalized cell lines, engineered to produce and release GABA, modulate the development of behavioral seizures. *Exp Neurol* 161(2):481-9.
- Thompson K, Holm AM, Schousboe A, Popper P, Micevych P, Wasterlain C. 1998. Hippocampal stimulation produces neuronal death in the immature brain. *Neuroscience* 82(2):337-48.
- Thompson KW. 2005. Genetically engineered cells with regulatable GABA production can affect afterdischarges and behavioral seizures after transplantation into the dentate gyrus. *Neuroscience* 133(4):1029-37.
- Thompson KW, Suchomelova LM. 2004. Transplants of cells engineered to produce GABA suppress spontaneous seizures. *Epilepsia* 45(1):4-12.

- Turner DA, Shetty AK. 2003. Clinical prospects for neural grafting therapy for hippocampal lesions and epilepsy. *Neurosurgery* 52(3):632-44.
- Turski L, Ikonomidou C, Turski WA, Bortolotto ZA, Cavalheiro EA. 1989. Review: cholinergic mechanisms and epileptogenesis. The seizures induced by pilocarpine: a novel experimental model of intractable epilepsy. *Synapse* 3(2):154-71.
- Virley D, Ridley RM, Sinden JD, Kershaw TR, Harland S, Rashid T, French S, Sowinski P, Gray JA, Lantos PL, Hodges H. 1999. Primary CA1 and conditionally immortal MHP36 cell grafts restore conditional discrimination learning and recall in marmosets after excitotoxic lesions of the hippocampal CA1 field. *Brain* 122(Pt 12):2321-35.
- Vonck K, Boon P, Achten E, De RJ, Caemaert J. 2002. Long-term amygdalohippocampal stimulation for refractory temporal lobe epilepsy. *Ann Neurol* 52(5):556-65.
- Vonck K, Thadani VY, Gilbert K, Dedeurwaerdere S, De Groote L, De Herdt V, Goossens L, Gossiaux F, Achten E, Thiery E, Vingerhoets G, van Roost D, Caemaert J, De Reuck J, Roberts D, Williamson P, Boon P. 2004. Vagus nerve stimulation for refractory epilepsy: A transatlantic experience. *Journal of Clinical Neurophysiology* 21(4):283-9.
- Weiss S, Dunne C, Hewson J, Wohl C, Wheatley M, Peterson AC, Reynolds BA. 1996. Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J Neurosci* 16(23):7599-609.
- Wernig M, Benninger F, Schmandt T, Rade M, Tucker KL, Bussow H, Beck H, Brustle O. 2004. Functional integration of embryonic stem cell-derived neurons in vivo. *J Neurosci* 24(22):5258-68.
- Westmoreland JJ, Hancock CR, Condie BG. 2001. Neuronal development of embryonic stem cells: a model of GABAergic neuron differentiation. *Biochem Biophys Res Commun* 284(3):674-80.
- Whittemore SR, Onifer SM. 2000. Immortalized neural cell lines for CNS transplantation. *Prog Brain Res* 127:49-65.
- Wichterle H, Lieberam I, Porter JA, Jessell TM. 2002. Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110(3):385-97.
- Wiebe S, Blume WT, Girvin JP, Eliasziw M. 2001. A randomized, controlled trial of surgery for temporal-lobe epilepsy. *N Engl J Med* 345(5):311-8.
- Wieser HG. 2004. Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis. *Epilepsia* 45(6):695-714.
- Yamashita T, Ninomiya M, Hernandez Acosta P, Garcia-Verdugo JM, Sunabori T, Sakaguchi M, Adachi K, Kojima T, Hirota Y, Kawase T, Araki N, Abe K, Okano H, Sawamoto K. 2006. Subventricular Zone-Derived Neuroblasts Migrate and Differentiate into Mature Neurons in the Post-Stroke Adult Striatum. *J Neurosci* 26(24):6627-36.
- Yilmazer-Hanke DM, Wolf HK, Schramm J, Elger CE, Wiestler OD, Blumcke I. 2000. Subregional pathology of the amygdala complex and entorhinal region in surgical specimens from patients with pharmacoresistant temporal lobe epilepsy. *J Neuropathol Exp Neurol* 59(10):907-20.
- Zaman V, Shetty AK. 2001. Fetal hippocampal CA3 cell grafts transplanted to lesioned CA3 region of the adult hippocampus exhibit long-term survival in a rat model of temporal lobe epilepsy. *Neurobiol Dis* 8(6):942-52.
- Zaman V, Shetty AK. 2003. Fetal hippocampal CA3 cell grafts enriched with fibroblast growth factor-2 exhibit enhanced neuronal integration into the lesioned aging rat hippocampus in a kainate model of temporal lobe epilepsy. *Hippocampus* 13(5):618-32.
- Zaman V, Turner DA, Shetty AK. 2000. Survival of grafted fetal neural cells in kainic acid lesioned CA3 region of adult hippocampus depends upon cell specificity. *Exp Neurol* 161(2):535-61.
- Zaman V, Turner DA, Shetty AK. 2001. Prolonged postlesion transplantation delay adversely influences survival of both homotopic and heterotopic fetal hippocampal cell grafts in Kainate-lesioned CA3 region of adult hippocampus. *Cell Transplant* 10(1):41-52.

Chapter 3

Adult Neurogenesis

INTRODUCTION ON ADULT NEUROGENESIS

DISCOVERY OF ADULT NEUROGENESIS

The dogma that neurogenesis, i.e. the birth of new neurons, is confined to a discrete period during development no longer stands. There is now clear evidence for persistent neurogenesis throughout life in two regions of the adult mammalian brain: the dentate gyrus of the hippocampus and the olfactory system. The first demonstration of adult neurogenesis was in a study done by Joseph Altman and Gopal D. Das. Those authors injected thymidine, radioactively labelled with tritium, to label dividing cells in adult rats. Once in the bloodstream, tritiated thymidine competes with endogenous thymidine in all cells in the S-phase of cell division and is permanently incorporated in the DNA. Using autoradiography Altman & Das identified labelled granule cells in the dentate gyrus of the hippocampus (Altman and Das, 1965). It took another 15 years for the next evidence on adult neurogenesis. Michael Kaplan used electron microscopy to proof the neuronal nature of cells labelled with tritiated thymidine (Kaplan and Hinds, 1977). In this latter study neurogenesis was demonstrated both in the hippocampus and in the olfactory bulbs.

In the early 90's technical advances, such as the use of 5-bromo-2 deoxyuridine (BrdU) or retroviral techniques to label dividing cells together with double-labelling immunohistochemistry and confocal microscopy, confirmed new neuron production in the dentate gyrus of the hippocampus (Cameron and others, 1993) and the olfactory bulbs with origin of the new neurons in the subventricular zone (SVZ) (Corotto and others, 1993; Luskin, 1993). Like tritiated thymidine, BrdU is a thymidine analogue that can be administrated systemically to label dividing cells. In contrast to tritiated thymidine, BrdU can be detected with immunohistochemistry, which allows the combination with two or more markers. Retroviral techniques depend on the local injection of retroviruses which can only infect dividing cells. Gene products from the incorporated viruses or viral genome can be detected at a later time in differentiated cells. Transduction of proliferating cells with a retrovirus expressing green fluorescent protein (GFP) even allows visualization of living cells which make studies on their functional properties possible. All these technical progresses have paved the path for further elaboration on the basic mechanisms and regulation of neurogenesis in both normal and pathological brain. In 1998, Peter S. Ericksson, a professor working at the Sahlgrenska University Hospital of Göteborg, discovered that neurogenesis also occurs in adult humans by analyzing the hippocampus of patients who had deceased from tongue or larynx cancer and had been injected with BrdU for diagnostic purposes (Eriksson and others, 1998).

In the following paragraphs we will give a short introduction on neurogenesis in the olfactory system and hippocampus of the normal adult brain. For more detailed information we refer to some excellent reviews (Kempermann and Gage, 1999; Gross, 2000; Lie and others, 2004; Ming and Song, 2005). It is very important to acknowledge that in both regions, neurogenesis comprises a dynamic process and is not a single event. Next to proliferation, it involves fate decision, migration, synaptic integration and survival.

NEUROGENESIS IN THE ADULT OLFACTORY SYSTEM

As already stated above, the origin of the new neurons in the adult olfactory bulbs is found in the SVZ (fig. 1).

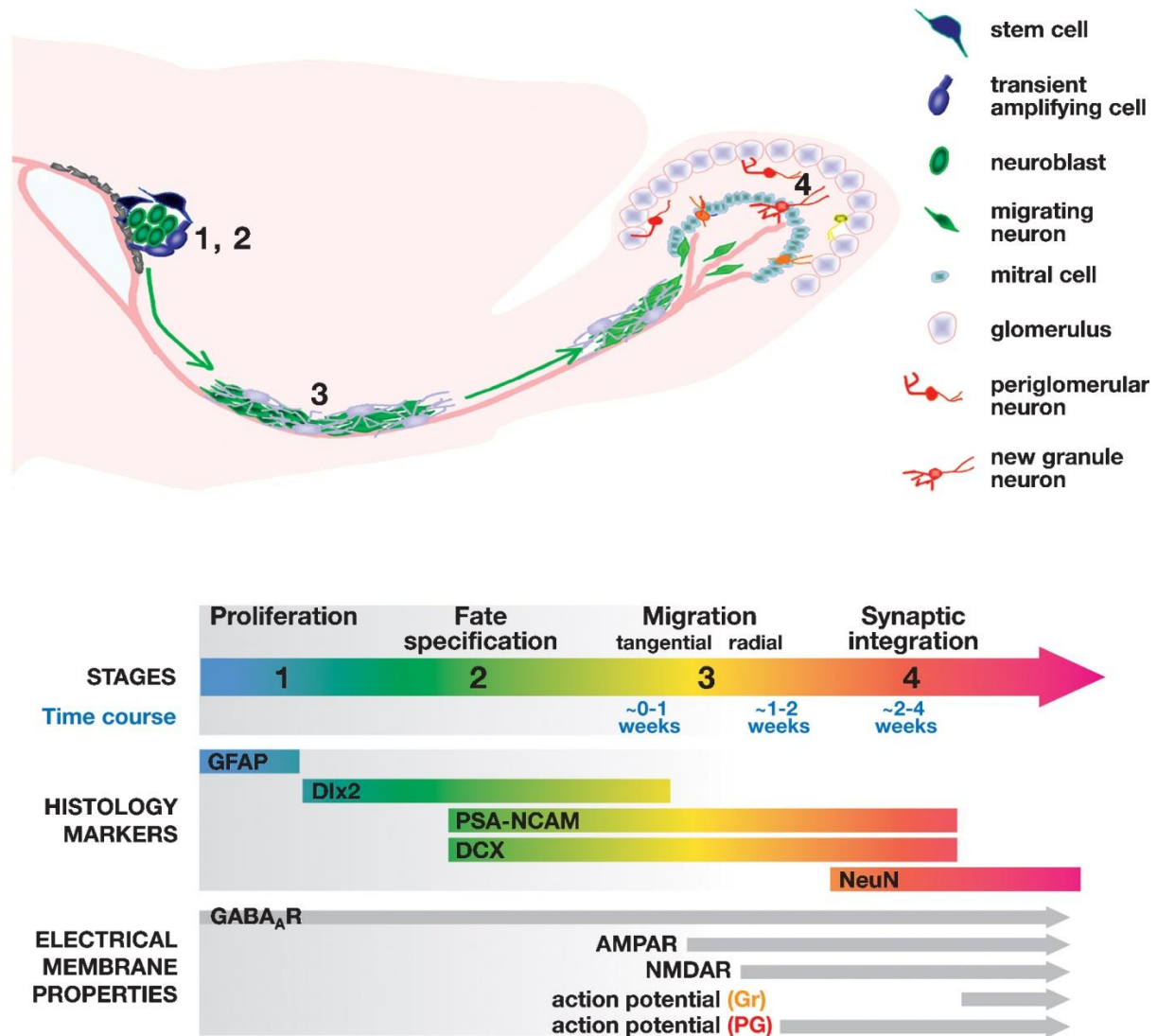


Fig 1: Schematic overview of the formation of new interneurons in the olfactory bulbs from NSC in the subventricular zone (SVZ). In this illustration four developmental stages are discriminated. Stage 1. Proliferation: astroglial-like stem cells (B-cells; GFAP⁺) give rise to transient amplifying cells (C-cells; GFAP⁺, Dlx2⁺). Stage 2. Fate specification: C-cells differentiate into young migrating neuroblasts (A-cells; PSA-NCAM⁺, DCX⁺). Stage 3. Migration: Neuroblasts migrate in chains through the rostral migratory stream (RMS) towards the olfactory bulb. Once reaching the bulb, new neurons migrate radially to the outer cell layers. Stage 4. Synaptic integration: Immature neurons differentiate into either granule neurons or periglomerular neurons. The specific properties of each stage are summarized below, mainly on the basis of studies in adult rodents. Figure modified from (Ming and Song, 2005)

In the SVZ, a subset of Glial Fibrillary Acidic Protein (GFAP)-positive astrocytes are believed to be the resident neural stem cells (NSC) (Doetsch and others, 1999; Imura and others, 2003). These astroglial-like NSC, termed B-cells, give rise to more rapidly dividing transiently amplifying progenitor cells, termed C cells (Doetsch, 2003). In the C-cells GFAP is no longer expressed but early neuronal transcription factors, such as distal-less homeobox2 (Dlx2) were found to be expressed in the C-cells (Doetsch and others, 2002). The C-cells, in their turn, give rise to young migrating neuroblasts, termed A-cells. Most of these A-cells express the polysialylated

form of the neural cell adhesion molecule (PSA-NCAM) and doublecortin (DCX), two molecules associated with neuronal migration. These neuroblasts migrate along the rostral migratory stream (RMS) towards the olfactory bulbs.

This migration along the RMS is a very unique process, called chain migration, in which dividing neuroblasts migrate closely associated with each other within a tube-like structure, formed by astrocytes (Lois and others, 1996). This astrocytic ribbon consists of slowly dividing cells which are presumed to have the same characteristics as the B-cells in the SVZ (Gritti and others, 2002). Based on removal and transplantation of the olfactory bulbs it is hypothesized that (unknown) chemoattractants in the olfactory bulbs are important driving forces for the directed migration of SVZ-derived neuroblasts (Liu and Rao, 2003). Another factor which seems to play an important role is Slit, a repulsant present in the striatum. From slit mutants it is evident that this factor prevents lateral migration of the neuroblasts into the striatum (Wu and others, 1999).

In migrating A-cells, expression of signs of mature neuronal phenotype is delayed until the cells have reached the olfactory bulbs. Once there, they become positive for neurotransmitters and their receptors and show electrophysiological signs of neuronal maturity (Carleton and others, 2003). The RMS terminates in the core of the olfactory bulbs where the neuroblasts switch to radial migration towards their target areas in the granule and the periglomerular cell layer. There they generate granule and periglomerular interneurons respectively. Although the majority of the newly generated neurons die, there are arguments that adult neurogenesis contributes to the growth of olfactory bulbs throughout life and leads to a partial neuronal turnover (Winner and others, 2002). Very recently, Eriksson and co-workers, described the presence of a RMS and the generation of new neurons in the olfactory bulb has also in humans (Curtis and others, 2007).

NEUROGENESIS IN THE ADULT HIPPOCAMPUS

In the adult hippocampus neurogenesis takes place in the dentate gyrus (fig. 2). As in the SVZ the NSC have astrocytic electrophysiological properties and resemble radial glia (Seri and others, 2001). The cells express the precursor cell marker nestin but also the astrocytic marker GFAP. These astroglial NSC (also called type 1 cells), with their cell bodies and vascular endfeet in the SGZ, have radial processes going through the granule cell layer (GCL) and short tangential processes extending along the border of the GCL and the hilus.

The type 1 astroglial stem cells slowly divide and give rise to transiently amplifying progenitor cells or type 2 cells. These type 2 cells can be divided in two groups: type 2a and type 2b cells. Type 2a cells have retracted their processes, still express nestin but are no longer positive for GFAP. Type 2b cells are still positive for nestin but also express DCX and PSA-NCAM. The highly proliferative type 2 cells give rise to type 3 cells which are no longer positive for nestin but still express PSA-NCAM and DCX (Kempermann and others, 2004). The type 3 cells become postmitotic and migrate radially into the GCL while they further develop to mature neurons (Hastings and Gould, 1999; van Praag and others, 2002; Kempermann and others, 2004). This stage of neuronal development is characterized by transient expression of calretinin, which is later exchanged for calbindin,

present in mature granule cells. During the calretinin phase the cells also express NeuN, the second known mature marker that persists in new neurons.

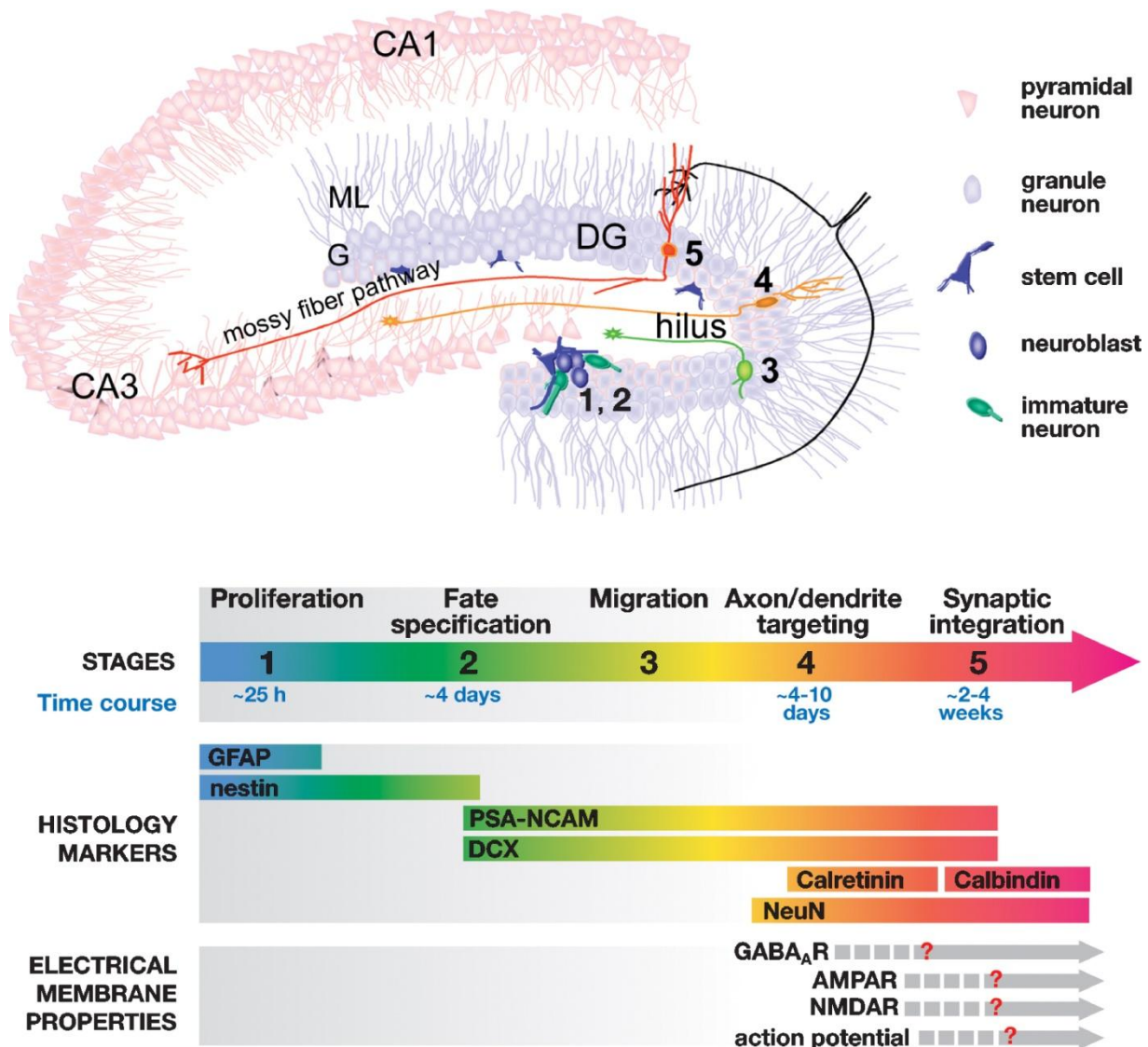


Fig 2: Schematic overview of the formation of new granule neurons in the granule cell layer (GCL) of the dentate gyrus from NSC in the subgranular zone (SGZ). In this illustration five developmental stages are discriminated. Stage 1. Proliferation: astroglial-like stem cells (type 1-cells; GFAP⁺, Nestin⁺) give rise to transient amplifying cells (type 2a-cells; GFAP⁺, Nestin⁺). Stage 2. Fate specification: Type 2a-cells give rise to migrating neuroblasts (type 3 cells; Nestin⁺, PSA-NCAM⁺, DCX⁺) over an intermediate phase (type 2b-cells; Nestin⁺, PSA-NCAM⁺, DCX⁺). Stage 3. Migration: Neuroblasts migrate a short distance in the GCL. Stage 4. Axon/dendrite targeting: Immature neurons extend their axonal projections along mossy fiber pathways to the CA3 pyramidal cell layer. They send their dendrites in the opposite direction towards the molecular layer. Stage 5. Synaptic integration: New granule neurons receive input from the entorhinal cortex and send outputs to the CA3 and hilus regions. The specific properties of each stage are summarized below, mainly on the basis of studies in adult rodents. Figure modified from (Ming and Song, 2005)

The first signs of neuronal function, in terms of the generation of action potentials and detectability of synapses, can be found when the cell still expresses nestin. As during embryogenesis GABAergic input precedes glutamatergic input (Ambrogini and others, 2004). The first axonal contact in the target CA3 region has been found as early as 3 to 5 days after division (Hastings and Gould, 1999). However, it takes 4 to 7 weeks for the new cells to become functionally indistinguishable from older granule cells (van Praag and others, 2002;

Jessberger and Kempermann, 2003). As in the olfactory bulbs the majority of the newborn neurons in the dentate gyrus of the hippocampus die. To give an idea: about 4800 surviving granule cells are generated per day in a C57BL/6 mouse at the age of 36 days. This number decreases strongly with age.

ADULT NEUROGENESIS AND NEURAL DAMAGE

NEUROGENIC AND NON-NEUROGENIC REGIONS

From both neurogenic regions (SVZ and hippocampus) neural stem cells can be isolated, expanded in culture and induced to generate both neurons and glial cells (Reynolds and others, 1992; Palmer and others, 1997). However, cells with the same *in vitro* properties have also been derived from other brain regions such as the caudal portions of the SVZ, the striatum, the neocortex, the optic nerve, the septum, the corpus callosum, the spinal cord, the substantia nigra, the retina and the hypothalamus (Palmer and others, 1995; Weiss and others, 1996; Shihabuddin and others, 1997; Palmer and others, 1999; Tropepe and others, 2000; Lie and others, 2002). This indicates that quiescent NSC also reside in other regions of the brain. Consequently it was investigated whether neurogenesis also occurs in those brain regions. There have been some reports which claim that neurogenesis occurs at low levels in the amygdala (Bernier and others, 2002), CA1 area of the hippocampus (Rietze and others, 2000), the dorsal vagal complex of the adult brainstem (Bauer and others, 2005), the spinal cord (Yamamoto and others, 2001a) and the substantia nigra (Zhao and others, 2003). However some of these results are still disputed (Kempermann and others, 1997; Horner and others, 2000; Lie and others, 2002; Frielingsdorf and others, 2004). Also in the neocortex extremely low levels of neurogenesis were reported and the newborn neurons only survived transiently (Gould and others, 2001). But again conflicting results exist (Kornack and Rakic, 2001; Koketsu and others, 2003). Overall, whether neurogenesis in intact adult brain takes place beyond hippocampus and olfactory bulbs remains highly controversial. Even if new neurons are generated in those “non-neurogenic” regions it is estimated to be in extremely low numbers.

A very likely, though not proven, explanation for the fact that neurogenesis only takes place in the olfactory bulbs and the granule cell layer and not in other regions containing neural progenitor cells is that the microenvironment needs to be permissive to sustain the formation and integration of newborn neurons. A lot of efforts have been made to unravel the factors which either promote or block neurogenesis in respectively the neurogenic and non-neurogenic region. Knowledge of these factors may contribute to strategies of brain repair through stimulation of endogenous neurogenesis.

One important factor for the modulation of precursor cell activity is thought to be cell-cell contact (Kempermann, 2006a). In both the SVZ and the hippocampus the astroglial-like NSC are in close contact with other astrocytes through gap junctions and with endothelial cells through vascular endfeet resting on basal membrane of the endothelial cells (Filippov and others, 2003).

Also diffusible molecules seem to regulate neurogenesis. Agasse and colleagues showed that factors secreted by SVZ cells promote neurogenesis *in vitro*, whereas protein factors from cortex actively inhibit neurogenesis

(Agasse and others, 2004). But when apoptosis was induced in the cortical explants, neurogenesis was promoted instead of blocked. Factors, which could play a role in this permissive and inhibitory signalling, are those which also play an important role in the regulation of neurogenesis and brain patterning during development. For example, it is known that bone morphogenic proteins (BMP) play an important role in patterning the embryo along the dorso-ventral axis. Reagents that block the BMP signalling pathway convert ectoderm effectively into neural tissue during embryogenesis. Lim and colleagues showed that blocking BMP signalling by ectopic expression of noggin allowed neurogenesis in the non-neurogenic striatum (Lim and others, 2000).

ADULT NEUROGENESIS IN PHOTOTOXIC LESION MODELS

In analogy to the *in vitro* study, performed by Agasse and colleagues, other studies have shown that the lack of neurogenic permissiveness in non-neurogenic regions may sometimes be overcome by a pathological stimulus in these brain regions. Pathology-induced neurogenesis can occur in two modalities: local dormant precursor cells may be activated to generate new neurons or precursor cells from normally neurogenic regions, e.g. the SVZ, may be attracted to migrate to the damaged sites.

In an elegant study, Magavi and coworkers, stereotactically injected a phototoxic drug into layer IV of the anterior cortex. Corticothalamic neurons in proximity to the lesion incorporated the drug and subsequent exposure to laser light induced death of these cells through apoptosis. The authors observed that doublecortin (DCX) positive precursor cells migrated towards the lesion. By labelling dividing cells with BrdU at the time of the lesion and staining for a neuronal marker up to 28 weeks after labelling they found that new neurons were generated at the lesion site. By retrograde labelling, using fluorogold, they confirmed that the cells adequately projected to the thalamus (Magavi and others, 2000). Using an analogue targeted apoptosis approach the same group showed that endogenous neuroblasts replace ablated corticospinal projection neurons in juvenile mice with a portion of these new neurons extending axons to the spinal cord (Chen and others, 2004). These studies are very promising as they show that cell death in non-neurogenic regions is sufficient to render the environment permissive for neurogenesis and that endogenous neural progenitors mediate cellular repair. However one needs to keep in mind that the damage in these models is very “sterile” and not a realistic model for brain injury.

ADULT NEUROGENESIS IN ISCHEMIC LESION MODELS

In models with more extensive damage, such as ischemia, neural replacement by endogenous neural precursors has also been demonstrated (fig. 3). Two independent studies showed that after inducing a focal ischemic lesion in the striatum by permanent occlusion of the middle cerebral artery, a small fraction (about 0.2%) of the lost medium spiny interneurons in the striatum was replaced (Arvidsson and others, 2002; Parent and others, 2002b). In a study done by Yamashita and colleagues, a cell-type-specific viral infection method was used to show that these new striatal neurons were derived from the GFAP-expressing NSC present in the

SVZ (fig. 3). The newborn cells migrated from the SVZ to the peri-infarct region in close association with the blood vessels (Yamashita and others, 2006).

In the four vessel occlusion (4-VO) model short and transient induction of global ischemia selectively affects hippocampal CA1 pyramidal neurons. In this model, Nakatomi and coworkers found that after the ischemic stimulus damaged CA1 neurons were replaced by endogenous neural progenitors (fig. 3). At least a portion of these progenitors arose from the posterior periventricular region (PPV). Brief intraventricular infusion of growth factors in the first week after stroke markedly increased reconstitution of CA1 (Nakatomi and others, 2002). In this model neuronal replacement was associated with partial behavioural recovery although the role of replaced neurons in functional improvement still needs to be proven.

In both the focal and global ischemia models, about a 10-fold increase in proliferation of dentate granule cell precursors has been observed. This resulted in a net increase of the number of newly generated granule neurons (fig. 3). As in normal animals, the granule neurons generated in response to the ischemic lesion integrated into the GCL. The relevance of increased granule cell neurogenesis in response to ischemia is currently not yet clear (Liu and others, 1998; Scharfman and others, 2000; Kee and others, 2001; Tanaka and others, 2004; Matsumori and others, 2006; Lichtenwalner and Parent, 2006).

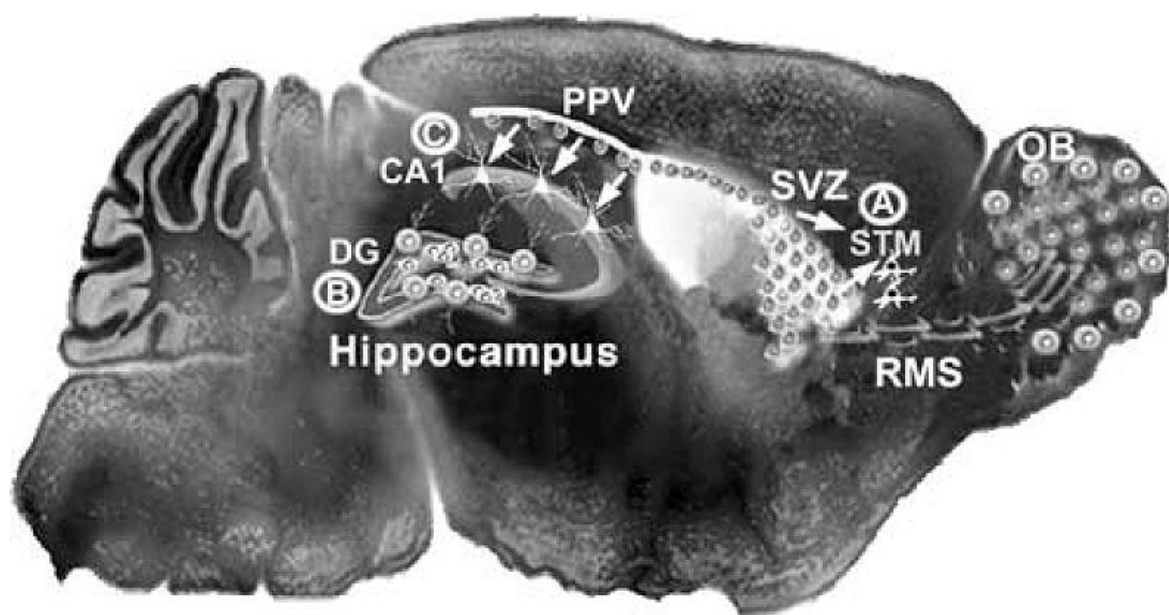


Fig 3: Endogenous neurogenesis is stimulated in focal and global ischemia models. A) After focal ischemic damage to the striatum (STM) SVZ-derived neuroblasts migrate towards the STM where they replace at least in part lost striatal neurons; B) In both focal and global ischemia models dentate granule cell neurogenesis is stimulated in the dentate gyrus; C) after transient global ischemia neuroblasts from the posterior periventricular region (PPV) migrate towards damaged CA1 region where they replace lost pyramidal neurons (arrows). Abbreviations: DG, dentate gyrus; OB, olfactory bulb; PPV, posterior periventricular region; RMS, rostral migratory stream; STM, striatum; SVZ, subventricular zone. Figure adopted from (Lichtenwalner and Parent, 2006)

In 6-OHDA nigrostriatal lesion models for PD induced cell proliferation was evident in the substantia nigra but only glial cells were generated (Lie and others, 2002). In this lesion model also direct migration of SVZ precursor cells into the striatum was seen. However in contrast to an ischemic lesion where a low level of regenerative neurogenesis was constitutively found (Arvidsson and others, 2002), a low level of neuronal differentiation of the SVZ precursors cells was only evident after infusion with transforming growth factor- α (Fallon and others, 2000). In the neurogenic regions a reduced neurogenesis was found and no signs of induced regeneration (Hoglinger and others, 2004).

In a quinolinic-acid model of Huntington disease medium spiny neurons of the striatum are degenerated. In response to the lesion, progenitor cell migration through the RMS is enhanced for up to 30 days. Additionally, a fraction of the SVZ-derived precursor cells migrate into the lesioned striatum. However, the cells adopted exclusively a glial fate and no regeneration of medium spiny striatal neurons could be demonstrated (Gordon and others, 2007).

Traumatic injury comprises several types of cellular damage (axonopathies, ischemia, hypoxia, excitotoxicity) and therefore the neurogenic response to trauma is complex and highly variable. So far, trauma-induced functional neurogenesis beyond the neurogenic regions has not been described yet. Migration of SVZ-derived precursor cells towards the lesion is evident but the cells uniquely adopt a glial fate and thereby seem to contribute to the glial scar (Kernie and others, 2001; Salman and others, 2004). In a fluid percussion model of traumatic brain injury there is a net increase (4 to 5 fold) in neurogenesis in both the ipsilateral and contralateral hippocampus (Dash and others, 2001; Kernie and others, 2001). The trauma-induced neurogenic response in the dentate gyrus is reduced in FGF-2-deficient mice and can be stimulated by gene transfer with FGF-2 (Yoshimura and others, 2003). Although percussion trauma also induces an increase in proliferation of SVZ precursors it is not yet clear whether this also lead to enhanced neurogenesis in the olfactory bulb.

Adult spinal cord has been demonstrated to contain multipotent precursor cells which can generate neurons after implantation into neurogenic regions. In case of injury to the spinal cord there is increased proliferation of the precursor cells. Most studies described that these neural precursors primarily generate astrocytes response to the lesion (e.g. (Yamamoto and others, 2001b; Takahashi and others, 2003)). A recent study, though, demonstrated that transduction of endogenous neural precursor cells with a neurogenic transcription factor (Neurgenin2) and treatment with a growth factor (BDNF) could induce in situ neuronal differentiation of the spinal cord NSC (Ohori and others, 2006).

Taken together, besides in stroke models, evidence for neuronal cell replacement from endogenous precursor cells at the lesion site is extremely limited. However, an increasing amount of studies show that modulation of the damaged environment in a variety of lesions by growth factor infusion, cell transplantation, gene transfer

can stimulate neuronal replacement from endogenous sources (Fallon and others, 2000; Nakatomi and others, 2002; Yoshimura and others, 2003; Ohori and others, 2006).

NEUROGENESIS IN EPILEPSY

Many intrinsic (e.g. aging, hormones, neurotransmitters, growth factors) and extrinsic (e.g. environmental enrichment, exercise, stress) factors influence the individual stages of adult neurogenesis. However also pathological situations, such as mechanical lesions, excitotoxicity, ischemia, traumatic brain injury and seizures, affect different stages of neurogenesis (Gould and Tanapat, 1997; Parent and others, 1997; Dash and others, 2001; Kee and others, 2001; Parent, 2002).

Seizure-activity, in both the kindling and SE models, has a profound influence on the process of neurogenesis in both the olfactory system and the dentate gyrus. In the SVZ, precursor cell proliferation is stimulated both after amygdala kindling (Sato and others, 2002) and pilocarpine (PILO)-induced SE (Parent and others, 2002a). In case of PILO-induced SE, migration of SVZ-derived progenitor cells along the rostral migratory stream (RMS) to the olfactory bulbs is enhanced (Parent and others, 2002a). Whether this higher number of migrating neuroblasts also results in more surviving olfactory bulb neurons is not yet elucidated.

Several studies in kindling and SE models have shown that seizure-activity significantly affects different stages of adult hippocampal neurogenesis (fig. 5) (Parent and others, 1997; Bengzon and others, 1997; Gray and Sundstrom, 1998; Parent and others, 1998; Scott and others, 1998; Scharfman and others, 2000; Nakagawa and others, 2000; Ferland and others, 2002; Romcy-Pereira and Garcia-Cairasco, 2003; Huttman and others, 2003; Mohapel and others, 2004; Jessberger and others, 2005; Smith and others, 2005; Pierce and others, 2005; Shapiro and others, 2005; Shapiro and Ribak, 2005; Parent and others, 2006; Overstreet-Wadiche and others, 2006; Shapiro and Ribak, 2006; Bonde and others, 2006). Seizure-activity in both models has clear stimulating effects on the proliferation of hippocampal radial glia-like stem and type 2 and type 3 progenitor cells (Parent and others, 1997; Bengzon and others, 1997; Gray and Sundstrom, 1998; Parent and others, 1998; Scott and others, 1998; Huttman and others, 2003; Jessberger and others, 2005; Smith and others, 2005). This results in a net increase of neurogenesis. In the chemoconvulsant and electrical SE models, precursor cell proliferation is increased five- to tenfold after a latent period of a few days (Parent and others, 1997; Gray and Sundstrom, 1998; Parent, 2002; Jessberger and others, 2005). In the amygdala kindling model and the rapid hippocampal kindling model, neurogenesis is increased respectively three to six times. In case of amygdala kindling increases are only seen after nine stage IV/V seizures while in the hippocampal kindling model neurogenesis is already increased three-fold five days after experiencing one single seizure (Bengzon and others, 1997). In models where kindling seizures are evoked by flurothyl or high intensity sound, no neural cell damage is evident but there is an upregulation of neurogenesis to the same extent as in electrical kindling models. This indicates that increased neurogenesis is caused by the seizure itself and not by seizure-induced damage (Ferland and others, 2002; Romcy-Pereira and Garcia-Cairasco, 2003). This increase in neurogenesis after seizures is only temporary

and returns back to baseline or even below within the first month after the seizures (Parent and others, 1997; Nakagawa and others, 2000).

Next to this increased rate of precursor cell proliferation and neurogenesis, seizure activity can also modulate the migration of neuroblasts (fig. 4). After kindling, but more prominently after SE, a part of the newborn neurons migrate aberrantly from the SGZ to ectopic locations in the molecular layer and the hilus of the dentate gyrus where they differentiate towards mature granule neurons (Parent and others, 1997; Scharfman and others, 2000; Parent, 2002; Smith and others, 2005; Pierce and others, 2005; Shapiro and others, 2005; Shapiro and Ribak, 2005; Parent and others, 2006; Shapiro and Ribak, 2006). Although these ectopic neurons demonstrate classic granule cell membrane properties and firing behaviour, they might contribute to the formation of hyperexcitable networks since they have an increased proportion of somatic and dendritic asymmetric (presumably excitatory) synapses (Dashtipour and others, 2003; Pierce and others, 2005), increased mossy fiber innervation (Pierce and others, 2005) and a distinct pattern of activation during spontaneous seizures. Furthermore, spontaneous epileptiform bursts have been recorded from these ectopic hilar granule cells, but not from conventional granule cells (Scharfman and others, 2000).

Synaptic integration of the newborn granule neurons can also be altered (fig. 4). Morphological analysis shows that part of the newborn granule cells have a persistent basal dendrite, which is normally only temporarily present in migrating immature neurons (Pierce and others, 2005; Shapiro and others, 2005; Shapiro and Ribak, 2005; Shapiro and Ribak, 2006).

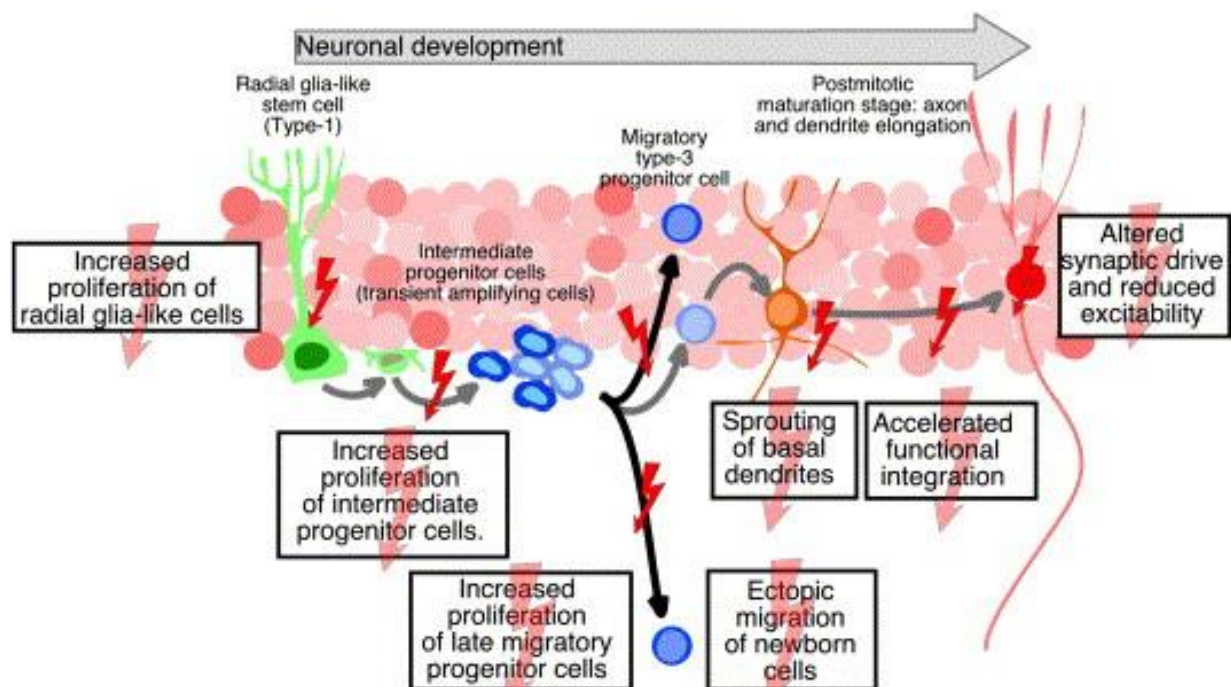


Fig 4: Schematic illustration of the influence of seizure-activity on the different stages of adult hippocampal neurogenesis. These influences range from increased proliferation of neural stem and progenitor cells to migration defects leading to ectopically positioned new granule cells. Also synaptic integration of the new cells is altered. The new neurons might show abnormal growth of basal dendrites and contribute to mossy fiber sprouting. Also their functional integration is sped up. However it seems that mature seizure-generated granule neurons receive reduced excitatory drive and enhanced inhibitory input. Figure adopted from (Kempermann, 2006b).

This basal dendrite projects into the hilus and is innervated by mossy fibers (Pierce and others, 2005; Shapiro and Ribak, 2006). Parent and coworkers have demonstrated that part of the newly born neurons form axonal collaterals which innervate neighbouring granule cells instead of CA3 or hilar neurons (Parent and others, 1997). This process is called mossy fiber sprouting and is described more extensively higher in this dissertation. The morphological development of immature granule cells is accelerated after seizures. The extension and arborisation of dendrites is sped up and the newborn neurons receive more rapidly excitatory input from appropriate (perforant path) but also from pathological afferents (sprouted mossy fibers) (Overstreet-Wadiche and others, 2006).

Although most changes in hippocampal neurogenesis in case of seizures suggest a pro-epileptic role, a recent study has shown that epilepsy-generated mature granule neurons in the GCL are suppressed, as they receive less excitatory drive and more inhibitory input compared to new granule neurons generated in physiological conditions (Jakubs and others, 2006). This observation suggests that the new neurons adjust their functional status to their local network environment and therefore might help to compensate for pathological excitation in epileptic seizures.

Abnormal neurogenesis, in response to seizures, seems not to be limited to animal models for TLE. In human TLE patients a higher number of neural precursors (Crespel and others, 2005; Thom and others, 2005) and the presence of putative ectopic granule cells (Parent and others, 2006) have been reported. Up till now, it is still not clear whether this seizure-induced alteration of neurogenesis is an attempt of the brain to repair neural damage or whether it is a part of the pathogenesis of TLE.

REFERENCES

- Agasse F, Roger M, Coronas V. 2004. Neurogenic and intact or apoptotic non-neurogenic areas of adult brain release diffusible molecules that differentially modulate the development of subventricular zone cell cultures. *European Journal of Neuroscience* 19(6):1459-68.
- Altman J, Das GD. 1965. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol* 124(3):319-35.
- Ambrogini P, Lattanzi D, Ciuffoli S, Agostini D, Bertini L, Stocchi V, Santi S, Cuppini R. 2004. Morpho-functional characterization of neuronal cells at different stages of maturation in granule cell layer of adult rat dentate gyrus. *Brain Res* 1017(1-2):21-31.
- Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. 2002. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med* 8(9):963-70.
- Bauer S, Hay M, Amilhon B, Jean A, Moyse E. 2005. In vivo neurogenesis in the dorsal vagal complex of the adult rat brainstem. *Neuroscience* 130(1):75-90.
- Bengzon J, Kokaia Z, Elmer E, Nanobashvili A, Kokaia M, Lindvall O. 1997. Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. *Proc Natl Acad Sci U S A* 94(19):10432-7.
- Bernier PJ, Bedard A, Vinet J, Levesque M, Parent A. 2002. Newly generated neurons in the amygdala and adjoining cortex of adult primates. *Proc Natl Acad Sci U S A* 99(17):11464-9.
- Bonde S, Ekdahl CT, Lindvall O. 2006. Long-term neuronal replacement in adult rat hippocampus after status epilepticus despite chronic inflammation. *Eur J Neurosci* 23(4):965-74.
- Cameron HA, Woolley CS, McEwen BS, Gould E. 1993. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience* 56(2):337-44.
- Carleton A, Petreanu LT, Lansford R, varez-Buylla A, Lledo PM. 2003. Becoming a new neuron in the adult olfactory bulb. *Nat Neurosci* 6(5):507-18.
- Chen J, Magavi SSP, Macklis JD. 2004. Neurogenesis of corticospinal motor neurons extending spinal projections in adult mice. *PNAS* 101(46):16357-62.
- Corotto FS, Henegar JA, Maruniak JA. 1993. Neurogenesis persists in the subependymal layer of the adult mouse brain. *Neurosci Lett* 149(2):111-4.
- Crespel A, Rigau V, Coubes P, Rousset MC, de Bock F, Okano H, Baldy-Moulinier M, Bockaert J, Lerner-Natoli M. 2005. Increased number of neural progenitors in human temporal lobe epilepsy. *Neurobiology of Disease* 19(3):436-50.
- Curtis MA, Kam M, Nannmark U, Anderson MF, Axell MZ, Wikkelsö C, Holtas S, van Roon-Mom WM, Björk-Eriksson T, Nordborg C, Frisen J, Dragunow M, Faull RL, Eriksson PS. 2007. Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. *Science* 315(5816):1243-9.
- Dash PK, Mach SA, Moore AN. 2001. Enhanced neurogenesis in the rodent hippocampus following traumatic brain injury. *Journal of Neuroscience Research* 63(4):313-9.
- Dashtipour K, Wong AM, Obenaus A, Spigelman I, Ribak CE. 2003. Temporal profile of hilar basal dendrite formation on dentate granule cells after status epilepticus. *Epilepsy Res* 54(2-3):141-51.
- Doetsch F. 2003. The glial identity of neural stem cells. *Nat Neurosci* 6(11):1127-34.
- Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97(6):703-16.
- Doetsch F, Petreanu L, Caille I, Garcia-Verdugo JM, varez-Buylla A. 2002. EGF Converts Transit-Amplifying Neurogenic Precursors in the Adult Brain into Multipotent Stem Cells. *Neuron* 36(6):1021-34.
- Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH. 1998. Neurogenesis in the adult human hippocampus. *Nat Med* 4(11):1313-7.

- Fallon J, Reid S, Kinyamu R, Opole I, Opole R, Baratta J, Korc M, Endo TL, Duong A, Nguyen G, Karkehabadhi M, Twardzik D, Patel S, Loughlin S. 2000. In vivo induction of massive proliferation, directed migration, and differentiation of neural cells in the adult mammalian brain. *PNAS* 97(26):14686-91.
- Ferland RJ, Gross RA, Applegate CD. 2002. Increased mitotic activity in the dentate gyrus of the hippocampus of adult C57BL/6J mice exposed to the flurothyl kindling model of epileptogenesis. *Neuroscience* 115(3):669-83.
- Filippov V, Kronenberg G, Pivneva T, Reuter K, Steiner B, Wang LP, Yamaguchi M, Kettenmann H, Kempermann G. 2003. Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. *Mol Cell Neurosci* 23(3):373-82.
- Frielingsdorf H, Schwarz K, Brundin P, Mohapel P. 2004. No evidence for new dopaminergic neurons in the adult mammalian substantia nigra. *Proc Natl Acad Sci U S A* 101(27):10177-82.
- Gordon RJ, Tattersfield AS, Vazey EM, Kells AP, McGregor AL, Hughes SM, Connor B. 2007. Temporal profile of subventricular zone progenitor cell migration following quinolinic acid-induced striatal cell loss. *Neuroscience* 146(4):1704-18.
- Gould E, Tanapat P. 1997. Lesion-induced proliferation of neuronal progenitors in the dentate gyrus of the adult. *Neuroscience* 80(2):427-36.
- Gould E, Vail N, Wagers M, Gross CG. 2001. Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. *Proc Natl Acad Sci U S A* 98(19):10910-7.
- Gray WP, Sundstrom LE. 1998. Kainic acid increases the proliferation of granule cell progenitors in the dentate gyrus of the adult rat. *Brain Res* 790(1-2):52-9.
- Gritti A, Bonfanti L, Doetsch F, Caille I, varez-Buylla A, Lim DA, Galli R, Verdugo JM, Herrera DG, Vescovi AL. 2002. Multipotent neural stem cells reside into the rostral extension and olfactory bulb of adult rodents. *J Neurosci* 22(2):437-45.
- Gross CG. 2000. Neurogenesis in the adult brain: death of a dogma. *Nat Rev Neurosci* 1(1):67-73.
- Hastings NB, Gould E. 1999. Rapid extension of axons into the CA3 region by adult-generated granule cells. *Journal of Comparative Neurology* 413(1):146-54.
- Hoglinger GU, Rizk P, Muriel MP, Duyckaerts C, Oertel WH, Caille I, Hirsch EC. 2004. Dopamine depletion impairs precursor cell proliferation in Parkinson disease. *Nat Neurosci* 7(7):726-35.
- Horner PJ, Power AE, Kempermann G, Kuhn HG, Palmer TD, Winkler J, Thal LJ, Gage FH. 2000. Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord. *J Neurosci* 20(6):2218-28.
- Huttman K, Sadgrove M, Wallraff A, Hinterkeuser S, Kirchhoff F, Steinhäuser C, Gray WP. 2003. Seizures preferentially stimulate proliferation of radial glia-like astrocytes in the adult dentate gyrus: functional and immunocytochemical analysis. *European Journal of Neuroscience* 18(10):2769-78.
- Imura T, Kornblum HI, Sofroniew MV. 2003. The Predominant Neural Stem Cell Isolated from Postnatal and Adult Forebrain But Not Early Embryonic Forebrain Expresses GFAP. *J Neurosci* 23(7):2824-32.
- Jakubs K, Nanobashvili A, Bonde S, Ekdahl CT, Kokaia Z, Kokaia M, Lindvall O. 2006. Environment Matters: Synaptic Properties of Neurons Born in the Epileptic Adult Brain Develop to Reduce Excitability. *Neuron* 52(6):1047-59.
- Jessberger S, Kempermann G. 2003. Adult-born hippocampal neurons mature into activity-dependent responsiveness. *Eur J Neurosci* 18(10):2707-12.
- Jessberger S, Romer B, Babu H, Kempermann G. 2005. Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. *Exp Neurol* 196(2):342-51.
- Kaplan MS, Hinds JW. 1977. Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. *Science* 197(4308):1092-4.
- Kee NJ, Preston E, Wojtowicz JM. 2001. Enhanced neurogenesis after transient global ischemia in the dentate gyrus of the rat. *Experimental Brain Research* 136(3):313-20.

- Kempermann G. 2006a. *Neurogenic and Non-neurogenic Regions. Adult neurogenesis. Stem cells and neuronal development in the adult brain.* New York: Oxford University Press; p 226-54.
- Kempermann G. 2006b. *They are not too excited: the possible role of adult-born neurons in epilepsy.* *Neuron* 52(6):935-7.
- Kempermann G, Gage FH. 1999. *New nerve cells for the adult brain.* *Sci Am* 280(5):48-53.
- Kempermann G, Jessberger S, Steiner B, Kronenberg G. 2004. *Milestones of neuronal development in the adult hippocampus.* *Trends Neurosci* 27(8):447-52.
- Kempermann G, Kuhn HG, Gage FH. 1997. *More hippocampal neurons in adult mice living in an enriched environment.* *Nature* 386(6624):493-5.
- Kernie SG, Erwin TM, Parada LF. 2001. *Brain remodeling due to neuronal and astrocytic proliferation after controlled cortical injury in mice.* *J Neurosci Res* 66(3):317-26.
- Koketsu D, Mikami A, Miyamoto Y, Hisatsune T. 2003. *Nonrenewal of neurons in the cerebral neocortex of adult macaque monkeys.* *J Neurosci* 23(3):937-42.
- Kornack DR, Rakic P. 2001. *Cell proliferation without neurogenesis in adult primate neocortex.* *Science* 294(5549):2127-30.
- Lichtenwalner RJ, Parent JM. 2006. *Adult neurogenesis and the ischemic forebrain.* *J Cereb Blood Flow Metab* 26(1):1-20.
- Lie DC, Dzieczapolski G, Willhoite AR, Kaspar BK, Shults CW, Gage FH. 2002. *The adult substantia nigra contains progenitor cells with neurogenic potential.* *J Neurosci* 22(15):6639-49.
- Lie DC, Song H, Colamarino SA, Ming GL, Gage FH. 2004. *Neurogenesis in the adult brain: new strategies for central nervous system diseases.* *Annu Rev Pharmacol Toxicol* 44:399-421.
- Lim DA, Tramontin AD, Trevejo JM, Herrera DG, Garcia-Verdugo JM, Alvarez-Buylla A. 2000. *Noggin antagonizes BMP signaling to create a niche for adult neurogenesis.* *Neuron* 28(3):713-26.
- Liu G, Rao Y. 2003. *Neuronal Migration from the Forebrain to the Olfactory Bulb Requires a New Attractant Persistent in the Olfactory Bulb.* *J Neurosci* 23(16):6651-9.
- Liu J, Solway K, Messing RO, Sharp FR. 1998. *Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils.* *J Neurosci* 18(19):7768-78.
- Lois C, Garcia-Verdugo JM, Alvarez-Buylla A. 1996. *Chain migration of neuronal precursors.* *Science* 271(5251):978-81.
- Luskin MB. 1993. *Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone.* *Neuron* 11(1):173-89.
- Magavi SS, Leavitt BR, Macklis JD. 2000. *Induction of neurogenesis in the neocortex of adult mice.* *Nature* 405(6789):951-5.
- Matsumori Y, Hong SM, Fan Y, Kayama T, Hsu CY, Weinstein PR, Liu J. 2006. *Enriched environment and spatial learning enhance hippocampal neurogenesis and salvages ischemic penumbra after focal cerebral ischemia.* *Neurobiol Dis* 22(1):187-98.
- Ming GL, Song H. 2005. *Adult neurogenesis in the mammalian central nervous system.* *Annu Rev Neurosci* 28:223-50.
- Mohapel P, Ekdahl CT, Lindvall O. 2004. *Status epilepticus severity influences the long-term outcome of neurogenesis in the adult dentate gyrus.* *Neurobiol Dis* 15(2):196-205.
- Nakagawa E, Aimi Y, Yasuhara O, Tooyama I, Shimada M, McGeer PL, Kimura H. 2000. *Enhancement of progenitor cell division in the dentate gyrus triggered by initial limbic seizures in rat models of epilepsy.* *Epilepsia* 41(1):10-8.
- Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M. 2002. *Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors.* *Cell* 110(4):429-41.

- Ohori Y, Yamamoto Si, Nagao M, Sugimori M, Yamamoto N, Nakamura K, Nakafuku M. 2006. Growth Factor Treatment and Genetic Manipulation Stimulate Neurogenesis and Oligodendrogenesis by Endogenous Neural Progenitors in the Injured Adult Spinal Cord. *J Neurosci* 26(46):11948-60.
- Overstreet-Wadiche LS, Bromberg DA, Bensen AL, Westbrook GL. 2006. Seizures accelerate functional integration of adult-generated granule cells. *J Neurosci* 26(15):4095-103.
- Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH. 1999. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J Neurosci* 19(19):8487-97.
- Palmer TD, Ray J, Gage FH. 1995. FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Mol Cell Neurosci* 6(5):474-86.
- Palmer TD, Takahashi J, Gage FH. 1997. The adult rat hippocampus contains primordial neural stem cells. *Mol Cell Neurosci* 8(6):389-404.
- Parent JM. 2002. The role of seizure-induced neurogenesis in epileptogenesis and brain repair. *Epilepsy Res* 50(1-2):179-89.
- Parent JM, Elliott RC, Pleasure SJ, Barbaro NM, Lowenstein DH. 2006. Aberrant seizure-induced neurogenesis in experimental temporal lobe epilepsy. *Ann Neurol* 59(1):81-91.
- Parent JM, Janumpalli S, McNamara JO, Lowenstein DH. 1998. Increased dentate granule cell neurogenesis following amygdala kindling in the adult rat. *Neurosci Lett* 247(1):9-12.
- Parent JM, Valentin VV, Lowenstein DH. 2002a. Prolonged seizures increase proliferating neuroblasts in the adult rat subventricular zone-olfactory bulb pathway. *J Neurosci* 22(8):3174-88.
- Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM. 2002b. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol* 52(6):802-13.
- Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH. 1997. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci* 17(10):3727-38.
- Pierce JP, Melton J, Punsoni M, McCloskey DP, Scharfman HE. 2005. Mossy fibers are the primary source of afferent input to ectopic granule cells that are born after pilocarpine-induced seizures. *Exp Neurol* 196(2):316-31.
- Reynolds BA, Tetzlaff W, Weiss S. 1992. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J Neurosci* 12(11):4565-74.
- Rietze R, Poulin P, Weiss S. 2000. Mitotically active cells that generate neurons and astrocytes are present in multiple regions of the adult mouse hippocampus. *J Comp Neurol* 424(3):397-408.
- Romcy-Pereira RN, Garcia-Cairasco N. 2003. Hippocampal cell proliferation and epileptogenesis after audiogenic kindling are not accompanied by mossy fiber sprouting or fluoro-jade staining. *Neuroscience* 119(2):533-46.
- Salman H, Ghosh P, Kernie SG. 2004. Subventricular zone neural stem cells remodel the brain following traumatic injury in adult mice. *J Neurotrauma* 21(3):283-92.
- Sato K, Iwai M, Nagano I, Shoji M, Abe K. 2002. Temporal and spacial changes of BrdU immunoreactivity in amygdala kindling development. *Neurol Res* 24(6):593-6.
- Scharfman HE, Goodman JH, Sollas AL. 2000. Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. *J Neurosci* 20(16):6144-58.
- Scott BW, Wang S, Burnham WM, De Boni U, Wojtowicz JM. 1998. Kindling-induced neurogenesis in the dentate gyrus of the rat. *Neurosci Lett* 248(2):73-6.
- Seri B, Garcia-Verdugo JM, McEwen BS, varez-Buylla A. 2001. Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J Neurosci* 21(18):7153-60.

- Shapiro LA, Korn MJ, Ribak CE. 2005. Newly generated dentate granule cells from epileptic rats exhibit elongated hilar basal dendrites that align along GFAP-immunolabeled processes. *Neuroscience* 136(3):823-31.
- Shapiro LA, Ribak CE. 2005. Integration of newly born dentate granule cells into adult brains: hypotheses based on normal and epileptic rodents. *Brain Res Brain Res Rev* 48(1):43-56.
- Shapiro LA, Ribak CE. 2006. Newly born dentate granule neurons after pilocarpine-induced epilepsy have hilar basal dendrites with immature synapses. *Epilepsy Res* 69(1):53-66.
- Shihabuddin LS, Ray J, Gage FH. 1997. FGF-2 is sufficient to isolate progenitors found in the adult mammalian spinal cord. *Exp Neurol* 148(2):577-86.
- Smith PD, Mclean KJ, Murphy MA, Turnley AM, Cook MJ. 2005. Seizures, not hippocampal neuronal death, provoke neurogenesis in a mouse rapid electrical amygdala kindling model of seizures. *Neuroscience* 136(2):405-15.
- Takahashi M, Arai Y, Kurosawa H, Sueyoshi N, Shirai S. 2003. Ependymal cell reactions in spinal cord segments after compression injury in adult rat. *J Neuropathol Exp Neurol* 62(2):185-94.
- Tanaka R, Yamashiro K, Mochizuki H, Cho N, Onodera M, Mizuno Y, Urabe T. 2004. Neurogenesis After Transient Global Ischemia in the Adult Hippocampus Visualized by Improved Retroviral Vector. *Stroke* 35(6):1454-9.
- Thom M, Martinian L, Williams G, Stoeber K, Sisodiya SM. 2005. Cell proliferation and granule cell dispersion in human hippocampal sclerosis. *J Neuropathol Exp Neurol* 64(3):194-201.
- Tropepe V, Coles BL, Chiasson BJ, Horsford DJ, Elia AJ, McInnes RR, van der KD. 2000. Retinal stem cells in the adult mammalian eye. *Science* 287(5460):2032-6.
- van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. 2002. Functional neurogenesis in the adult hippocampus. *Nature* 415(6875):1030-4.
- Weiss S, Dunne C, Hewson J, Wohl C, Wheatley M, Peterson AC, Reynolds BA. 1996. Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J Neurosci* 16(23):7599-609.
- Winner B, Cooper-Kuhn CM, Aigner R, Winkler J, Kuhn HG. 2002. Long-term survival and cell death of newly generated neurons in the adult rat olfactory bulb. *European Journal of Neuroscience* 16(9):1681-9.
- Wu W, Wong K, Chen J, Jiang Z, Dupuis S, Wu JY, Rao Y. 1999. Directional guidance of neuronal migration in the olfactory system by the protein Slit. *Nature* 400(6742):331-6.
- Yamamoto S, Yamamoto N, Kitamura T, Nakamura K, Nakafuku M. 2001a. Proliferation of parenchymal neural progenitors in response to injury in the adult rat spinal cord. *Exp Neurol* 172(1):115-27.
- Yamamoto Si, Yamamoto N, Kitamura T, Nakamura K, Nakafuku M. 2001b. Proliferation of Parenchymal Neural Progenitors in Response to Injury in the Adult Rat Spinal Cord. *Experimental Neurology* 172(1):115-27.
- Yamashita T, Ninomiya M, Hernandez Acosta P, Garcia-Verdugo JM, Sunabori T, Sakaguchi M, Adachi K, Kojima T, Hirota Y, Kawase T, Araki N, Abe K, Okano H, Sawamoto K. 2006. Subventricular Zone-Derived Neuroblasts Migrate and Differentiate into Mature Neurons in the Post-Stroke Adult Striatum. *J Neurosci* 26(24):6627-36.
- Yoshimura S, Teramoto T, Whalen MJ, Irizarry MC, Takagi Y, Qiu J, Harada J, Waeber C, Breakefield XO, Moskowitz MA. 2003. FGF-2 regulates neurogenesis and degeneration in the dentate gyrus after traumatic brain injury in mice. *J Clin Invest* 112(8):1202-10.
- Zhao M, Momma S, Delfani K, Carlen M, Cassidy RM, Johansson CB, Brismar H, Shupliakov O, Frisen J, Janson AM. 2003. Evidence for neurogenesis in the adult mammalian substantia nigra. *Proc Natl Acad Sci U S A* 100(13):7925-30.

Chapter 4

Rationale, aims and outline of the thesis

RATIONALE

A recent study evaluated the effectiveness of AED treatment in a large patient group (2200 patients) and demonstrated that patients with TLE showed the worst prognosis. Only 20% of patients with TLE remained seizure free for longer than one year. In case of HS and dual pathology (HS and another lesion) seizures were controlled only in 11% and 3% respectively {Semah, 1998 377 /id}. This is in line with the current assessment that TLE is the most medically refractory form of human epilepsy. As newer AED do not differ markedly in efficacy from the more traditional ones, most medically refractory TLE patients are considered for epilepsy surgery {Spencer, 2002 27 /id}. A recent randomized controlled study has demonstrated the superior outcome of resective surgery compared to medical therapy. 58% of the TLE patients were seizure free for at least one year compared to 8% in the medical group {Wiebe, 2001 14 /id}. But there are a considerable number of patients in whom resective surgery cannot be performed because presurgical evaluation has revealed that seizures originate in both temporal lobes, or that there is a risk for substantial decline in memory after temporal lobectomy. Only 30% to 50% of the surgical candidates who are enrolled in a presurgical evaluation protocol eventually undergo surgery {Boon, 1999 17 /id}. One of the major side effects of resective surgery in the temporal lobe is reported to be an acceleration of cognitive decline, especially in those patients in which presurgical memory and MRI was normal and surgery did not lead to a control of seizures {Helmstaedter, 2003 378 /id}. Hoping to minimize the risk of memory loss, less invasive surgical procedures and smaller resections e.g. amygdalohippocampectomy have been adopted. Unfortunately, there is evidence of memory decline even with these more limited resections {Gleissner, 2002 379 /id}. The inability to adequately treat all refractory MTLE patients with resective surgery and the reported risk of cognitive decline after resection of temporal lobe structures provides a continuous impetus to search for non-resective treatments which are able to control or even cure MTLE with the preservation or even restoration of cognitive functions.

Neurostimulation is one of the recent developments in the treatment of epilepsy. Two forms of neurostimulation are particularly interesting for the treatment of epilepsy, namely vagus nerve stimulation (VNS) and deep brain stimulation (DBS).

To date, only VNS is an approved neurostimulation therapy for epilepsy. Two prospective multicentre double-blind studies have demonstrated the efficacy of VNS by reporting a reduction in seizure frequency of respectively 24 % {1995 380 /id} and 28% {Handforth, 1998 319 /id}. In an open label study performed at Ghent University Hospital and Dartmouth Hitchcock Medical Centre a reduction in seizure frequency of 55% was demonstrated {Vonck, 2004 24 /id}. Unfortunately, the number of patients rendered seizure-free (without medication) with VNS is low.

DBS of CNS structures such as the anterior and centromedian nuclei of the thalamus, the caudate nucleus, the subthalamic nucleus and the hippocampus is a more invasive treatment option. Multicentre studies are in progress to assess the value of DBS of the anterior thalamus and subthalamic nucleus {Loddenkemper, 2001 381 /id}. An open label trial performed at Ghent University Hospital has demonstrated the efficacy and safety

of DBS of medial temporal lobe structures with a significant reduction of epileptic seizures and epileptic spikes {Vonck, 2002 26 /id}. Up till now only one randomized controlled study has been performed, with DBS focussed to the anterior thalamic nucleus, but this study did not confirm efficacy {Fisher, 1992 382 /id}. Since the precise mechanism of action of for both neurostimulation techniques (VNS, DBS) is not yet elucidated, it is difficult to adjust stimulation parameters or to identify ideal treatment candidates in order to optimize outcome.

Because even after VNS or DBS a lot of patients remain refractory, preclinical studies are ongoing to develop new treatments for refractory TLE patients. A possible alternative treatment option for TLE is cell therapy in which cells are used to mediate structural and/or neurochemical repair of hyperexcitable networks. There are two main approaches in which cell therapy could be applied to treat TLE. In a first approach, exogenous cells are transplanted into the epileptic brain, with or without previous manipulation *in vitro*. In a second approach, endogenous cell replacement is manipulated to reduce hyperexcitability.

The first type of cells that were transplanted into different brain regions of various TLE models were fetal brain cells. A review of these experiments is given in the introduction. In general, transplantation of fetal hippocampal cells in damaged areas of the hippocampus resulted in the partial replacement of lost neurons, the restoration of some appropriate synaptic connections and the partial reversal of some biochemical abnormalities {Turner, 2003 383 /id}. Alternatively, fetal cells were derived from regions containing high amounts of neurotransmitters involved in endogenous inhibitory mechanisms (NA, AchE, and GABA) and transplanted into the epileptogenic zone or areas that regulate seizure threshold and/or propagation prior to kindling or before the induction of acute seizures. In many cases this resulted into an increased threshold to evoke seizures, a reduction in seizure severity and/or a decrease in kindling rate {Nilsen, 2004 384 /id;Raedt, 2007 385 /id}. The effect of fetal cell transplantation on spontaneous seizures was rarely studied.

Although the results were very promising, clinical applications on a large scale will always be hampered by ethical and practical problems associated with the use of fetal tissue. One of the major problems is the inability to expand or store fetal tissue which implies that a high amount of fetuses are needed to obtain sufficient tissue for transplantation.

In analogy to cell therapy for other neurodegenerative diseases, it is mandatory that we search for alternative donor cells to replace fetal tissue {Lindvall, 2004 267 /id}. Great promise holds for stem and/or progenitor cells to replace fetal cells for the repair of damaged neural networks. As described in the introduction, stem and progenitor cells with neuropotency can be isolated from the blastocyst (embryonic stem cells), embryonic and fetal brain tissue, adult neural tissue and adult non-neural tissues. Especially the potential for self-renewal and differentiation towards neural cell types make neuropotent stem/progenitor cells potentially useful for transplantation in a variety of different neurological diseases such as Parkinson's disease, stroke, Huntington's disease, epilepsy, etc. Particularly interesting for future use in a clinical setting are neuropotent adult-derived (somatic) stem cells since these do not involve ethical issues and would make autologous transplantation possible, free of immunorejection problems.

Neural stem cells (NSC) isolated from adult CNS regions (hippocampus, SVZ, spinal cord, etc.) but also stem cells-derived from adult non-neural tissue such as skin, adipose tissue, liver and bone marrow were reported to express neural markers after *in vitro* differentiation. However, conflicting reports in literature {Bertani, 2005 386 /id;Lu, 2005 387 /id;Neuhuber, 2004 388 /id} motivated us to compare the *in vitro* neurogenic potential of bone marrow-derived multipotent stem cells, called multipotent adult progenitor cells (MAPC), to that of brain-derived neural stem cells {Raedt, 2006 634 /id}.

Alternative cell sources have been transplanted into the brain of TLE models with promising antiepileptic and anti-epileptogenic effects. Fibroblasts {Huber, 2001 312 /id}, myoblasts {Guttinger, 2005 368 /id}, embryonic stem cells and embryonic stem cell-derived glia {Guttinger, 2005 369 /id}, engineered to release high amounts of adenosine were encapsulated in semi-permeable polymers and transplanted in the lateral ventricle of fully kindled rats. This resulted in a robust suppression of kindling evoked seizures {Guttinger, 2005 368 /id;Guttinger, 2005 369 /id;Huber, 2001 312 /id}. Grafting of conditionally immortalized neuroprogenitor cells, isolated from mouse embryonic cortex and engineered to release high amounts of GABA, increased kindling threshold and suppressed kindling rate after transplantation in the substantia nigra, the hippocampus and the pyriform cortex {Gernert, 2002 306 /id;Thompson, 2000 309 /id;Thompson, 2005 363 /id}. These GABA releasing cells were also grafted in rats with spontaneous epileptic seizures and had a significant suppression of epileptic spikes and seizures as a result {Thompson, 2004 310 /id}. However, up till now, no long term antiepileptic effects after transplantation of adenosine- or GABA-releasing cells could be demonstrated due to progressive cell death of the transplanted cells, including those that did not secrete the therapeutic agent {Fedele, 2004 311 /id;Guttinger, 2005 369 /id;Huber, 2001 312 /id;Thompson, 2005 363 /id}. So in order to obtain more robust antiepileptic effects, other cell types and/or transplantation paradigms need to be tested in order to maximize cell survival and suppression of evoked or spontaneous seizures.

In this dissertation, we transplanted adult-derived NSC in the damaged hippocampus of the intrahippocampal KA model for TLE. Since habitual seizure parameters were not yet determined for this model, we first monitored this model for an extended time using video-EEG monitoring to assure its future usefulness for testing of seizure-suppressive effects of antiepileptic therapies, such as cell transplantation. Consequently, we set up a transplantation experiment to assess the influence of several variables (such as the presence of a KA lesion, delay between lesion and transplantation and delay between transplantation and tissue examination) on different outcome parameters (such as survival, dispersion and differentiation).

A second cell therapy approach involves modulation of endogenous neurogenesis. Endogenous neurogenesis is reported to be stimulated after different types of brain insults such as seizures, stroke and trauma. In stroke, endogenous neuroprogenitor cells of the SVZ migrate towards the damaged striatum and hippocampus where they replace respectively lost striatal and hippocampal neurons {Bendel, 2005 389 /id;Parent, 2003 390 /id;Yagita, 2001 391 /id;Parent, 2002 38 /id}. This endogenous repair can be stimulated by perfusion of growth factors in the ventricles following stroke {Nakatomi, 2002 263 /id}. Neurogenesis is also increased after seizures but whether this is a reflection of endogenous repair, a contribution to the formation of epileptic networks or a

bystander effect of increased neural activity is not yet elucidated. Especially in the hippocampus, there are reasons to believe that aberrant neurogenesis in response to seizures could contribute to epileptogenesis. Firstly, increased neurogenesis and epileptogenesis seem to occur during the same time window {Parent, 1997 125 /id;Smith, 2005 132 /id}. Secondly, synaptic integration of newborn neurons is accelerated and enhanced in response to seizures {Overstreet-Wadiche, 2006 129 /id}. Thirdly, in contrast to normal situations part of the newborn neurons form a persistent basal dendrite on which they receive excitatory inputs and they form axons which innervate neighboring excitatory neurons {Shapiro, 2005 136 /id}. Fourthly, some newborn granule neurons seem to contribute to the formation of recurrent excitatory networks by their involvement in mossy fiber sprouting {Parent, 1997 125 /id}. Finally, part of the newborn neurons migrates towards ectopic location in the hilus in proximity to the CA3 layer. This abnormal location could predispose the cells to contribute to hyperexcitable networks. For example, they exhibit abnormal spontaneous network bursts after pilocarpine-induced status epilepticus {Scharfman, 2000 134 /id}. They receive dense mossy fiber innervation {Pierce, 2005 133 /id} and they are likely to be innervated by CA3 pyramidal cells because they exhibit closely synchronized burst discharges in slices from pilocarpine-treated rats {Scharfman, 2000 134 /id}.

Based on these indications for a contributing role of aberrant neurogenesis in the formation of hyperexcitable networks, we have explored the possibility to block seizure induced increases in neurogenesis using low-dose, whole brain radiation in order to interfere with epileptogenesis in a kindling model for TLE.

The aims of this dissertation were to test the feasibility of two different cell therapy approaches for TLE: cell transplantation and modulation of endogenous neurogenesis. The following questions were raised:

CELL TRANSPLANTATION FOR TEMPORAL LOBE EPILEPSY:

A. *Neuropotency of multipotent adult progenitor-like cells and adult neural stem cells*

1. Can we isolate cell clones from the bone marrow which display the high proliferative potential, morphology and phenotypic profile as described for multipotent adult progenitor cells (MAPC)?
2. Are the MAPC-like cells pluripotent? In other words can we differentiate them towards endodermal (endothelium), mesodermal (hepatocytes) and ectodermal (neuroepithelium) cells?
3. Can we isolate neurosphere-forming neural stem cell (NSC) clones from the SVZ of adult rats?
4. Can these NSC be differentiated towards the three neural lineages: neurons, oligodendrocytes and astrocytes?
5. Which *in vitro* differentiation protocols can be used to compare neuropotency of both MAPC-like cells and NSC?
6. Which techniques can be used to evaluate neuropotency?
7. Is there a difference in the neuropotency of MAPC-like cells and NSC?

B. *Intrahippocampal kainic acid model for temporal lobe epilepsy*

1. What is the mortality rate after SE induced by intrahippocampal KA injection?
2. What is the fraction of rats displaying spontaneous seizures after intrahippocampal KA injection?
3. At what time following KA-induced SE can spontaneous seizures be detected?
4. What is the behavioural component of the spontaneous seizures?
5. What is the mean duration of the spontaneous seizures?
6. Is there a large variation in seizure frequency among rats?
7. Is there a progression in seizure frequency?
8. Do the spontaneous seizures occur in a circadian rhythm?

9. Could the intrahippocampal KA model be used for the evaluation of anti-epileptic effects of cell transplantation?

C. Transplantation of adult neural stem cells in the intrahippocampal kainic acid model

1. Do adult-derived NSC survive after transplantation in the intact or KA-lesioned hippocampus?
2. Is the survival rate affected due to the presence of the lesion?
3. Are the dispersion and/or integration of the transplanted cells different in intact compared to lesioned hippocampus?
4. To which type of cells do NSC differentiate after transplantation?
5. Is the cell fate of the NSC influenced by the presence of a KA lesion?
6. Are outcome parameters of the transplantation (survival, dispersion, integration and differentiation) affected by a longer delay between the lesion and the transplantation?
7. Is survival of the NSC stable over time?
8. Could NSC be used for the structural repair of damaged epileptic networks in the intrahippocampal kainic acid model?
9. Could NSC be used for the local delivery of seizure-modifying substances?

MODULATION OF ENDOGENOUS NEUROGENESIS

1. Can low-dose, whole brain radiation suppress neurogenesis in the rapid kindling model for TLE?
2. Is there a permanent suppression of neurogenesis?
3. Is there a difference in afterdischarge thresholds and afterdischarge duration between radiated and non-radiated rats?
4. Does radiation affect kindling rate?
5. Is brain inflammation different between radiated and non-radiated rats?
6. Can radiated rats be fully kindled?
7. Does radiation affect permanence of the fully kindled state?
8. Could suppression of seizure-induced neurogenesis be a potential cell therapy for TLE?

OUTLINE OF THE THESIS

Chapter 1 provides a general introduction on epilepsy, the treatment of epilepsy and animal models for epilepsy, all with a special focus on temporal lobe epilepsy (TLE).

Chapter 2 present two reviews. The first review more generally deals with cell therapy in different neurological diseases (Parkinson's disease, stroke and epilepsy). In an addendum the reader is updated with the most recent information concerning the items discussed in this first review. The second manuscript focuses specifically on cell therapy for TLE.

Chapter 3 provides a general introduction on neurogenesis and its modulation in response to different lesions and epileptic seizures.

Chapter 4 describes the rationale for testing the feasibility of cell transplantation and modulation of endogenous neurogenesis as potential cell therapies for TLE. Specific questions regarding both approaches that have been addressed in this dissertation are clearly formulated. Methods and results of our original research are reported in Chapter 3 and Chapter 4.

Chapter 5 focuses on the feasibility of cell transplantation as potential treatment of TLE. It is a compilation of three manuscripts. The first manuscript describes an *in vitro* experiment in which we compared the neuropotency of two adult-derived stem cell types: brain-derived neural stem cells and bone marrow-derived multipotent stem cells. The second manuscript describes a long term video-EEG monitoring study to characterize spontaneous seizures in the intrahippocampal kainic acid (KA) rat model for TLE. The third manuscript describes a study in which adult neural stem cells were transplanted in the sclerotic hippocampus of the intrahippocampal KA model for TLE.

Chapter 6 focuses on the feasibility of modulating endogenous neurogenesis as potential treatment for TLE. It comprises one study in which rats were exposed to low-dose, whole brain radiation in order to suppress neurogenesis during kindling epileptogenesis.

Chapter 7 discusses the results obtained from this dissertation. The different observations are put in the context of the current literature and the main questions and objectives, formulated in this thesis.

Chapter 8 provides a general conclusion by answering the research questions, formulated in chapter 2.

Chapter 9 provides future perspectives for further research within the field of cell transplantation and modulation of endogenous neurogenesis.

Finally a summary of this dissertation is presented in English, Dutch and French.

REFERENCES

- Bendel O, Bueters T, von EM, Ove OS, Sandin J, von EG, 2005. Reappearance of hippocampal CA1 neurons after ischemia is associated with recovery of learning and memory. *J Cereb. Blood Flow Metab* 25: 1586-1595.
- Bertani N, Malatesta P, Volpi G, Sonogo P, Perris R, 2005. Neurogenic potential of human mesenchymal stem cells revisited: analysis by immunostaining, time-lapse video and microarray. *J. Cell Sci.* 118: 3925-3936.
- Boon P, Vandekerckhove T, Achten E, Thiery E, Goossens L, Vonck K, D'Have M, Van Hoey G, Vanrumste B, Legros B, Defreyne L, De Reuck J, 1999. Epilepsy surgery in Belgium, the experience in Gent. *Acta Neurol Belg* 99: 256-65.
- Boon P, Vonck K, De Herdt V, Van Dycke A, Goethals M, Goossens L, Van Zandijcke M, De Smedt T, Dewaele I, Achten R, Wadman W, Dewaele F, Caemaert J, Van Roost D, 2007. Deep Brain Stimulation in Patients with Refractory Temporal Lobe Epilepsy. *Epilepsia*. In Press.
- Fedele DE, Koch P, Scheurer L, Simpson EM, Mohler H, Brustle O, Boison D, 2004. Engineering embryonic stem cell derived glia for adenosine delivery. *Neurosci. Lett.* 370: 160-165.
- Fisher RS, Uematsu S, Krauss GL, Cysyk BJ, McPherson R, Lesser RP, Gordon B, Schwerdt P, Rise M, 1992. Placebo-controlled pilot study of centromedian thalamic stimulation in treatment of intractable seizures. *Epilepsia* 33: 841-851.
- Gernert M, Thompson KW, Loscher W, Tobin AJ, 2002. Genetically engineered GABA-producing cells demonstrate anticonvulsant effects and long-term transgene expression when transplanted into the central piriform cortex of rats. *Exp Neurol* 176: 183-92.
- Gleissner U, Helmstaedter C, Schramm J, Elger CE, 2002. Memory Outcome after Selective Amygdalohippocampectomy: A Study in 140 Patients with Temporal Lobe Epilepsy. *Epilepsia* 43: 87-95.
- Guttinger M, Fedele D, Koch P, Padrun V, Pralong WF, Brustle O, Boison D, 2005a. Suppression of kindled seizures by paracrine adenosine release from stem cell-derived brain implants. *Epilepsia* 46: 1162-1169.
- Guttinger M, Padrun V, Pralong WF, Boison D, 2005b. Seizure suppression and lack of adenosine A1 receptor desensitization after focal long-term delivery of adenosine by encapsulated myoblasts. *Exp. Neurol.* 193: 53-64.
- Handforth A, DeGiorgio CM, Schachter SC, Uthman BM, Naritoku DK, Tecoma ES, Henry TR, Collins SD, Vaughn BV, Gilmartin RC, Labar DR, Morris GL, III, Salinsky MC, Osorio I, Ristanovic RK, Labiner DM, Jones JC, Murphy JV, Ney GC, Wheless JW, 1998. Vagus nerve stimulation therapy for partial-onset seizures: a randomized active-control trial. *Neurology* 51: 48-55.
- Helmstaedter C, Kurthen M, Lux S, Reuber M, Elger CE, 2003. Chronic epilepsy and cognition: a longitudinal study in temporal lobe epilepsy. *Ann. Neurol.* 54: 425-432.
- Huber A, Padrun V, Deglon N, Aebischer P, Mohler H, Boison D, 2001. Grafts of adenosine-releasing cells suppress seizures in kindling epilepsy. *Proc Natl Acad Sci U S A* 98: 7611-6.
- Lindvall O, Kokaia Z, Martinez-Serrano A, 2004. Stem cell therapy for human neurodegenerative disorders-how to make it work. *Nat. Med.* 10 Suppl: S42-S50.
- Loddenkemper T, Pan A, Neme S, Baker KB, Rezai AR, Dinner DS, Montgomery EB, Jr., Luders HO, 2001. Deep brain stimulation in epilepsy. *J Clin. Neurophysiol* 18: 514-532.
- Lu P, Tuszynski MH, 2005. Can bone marrow-derived stem cells differentiate into functional neurons? *Exp. Neurol.* 193: 273-278.

- Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M, 2002. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* 110: 429-441.
- Neuhuber B, Gallo G, Howard L, Kostura L, Mackay A, Fischer I, 2004. Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. *J. Neurosci. Res.* 77: 192-204.
- Nilsen KE, Cock HR, 2004. Focal treatment for refractory epilepsy: hope for the future? *Brain Res. Brain Res. Rev.* 44: 141-153.
- Overstreet-Wadiche LS, Bromberg DA, Bensen AL, Westbrook GL, 2006. Seizures accelerate functional integration of adult-generated granule cells. *J. Neurosci.* 26: 4095-4103.
- Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH, 1997. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci* 17: 3727-38.
- Parent JM, 2002. The role of seizure-induced neurogenesis in epileptogenesis and brain repair. *Epilepsy Res* 50: 179-89.
- Parent JM, 2003. Injury-induced neurogenesis in the adult mammalian brain. *Neuroscientist.* 9: 261-272.
- Pierce JP, Melton J, Punsoni M, McCloskey DP, Scharfman HE, 2005. Mossy fibers are the primary source of afferent input to ectopic granule cells that are born after pilocarpine-induced seizures. *Exp. Neurol.* 196: 316-331.
- Raedt R, Pinxteren J, VanDycke A, Waeytens A, Craeye D, Timmermans F, Vonck K, Vandekerckhove B, Plum J, Boon P, 2006. Differentiation assays of bone marrow-derived Multipotent Adult Progenitor Cell (MAPC)-like cells towards neural cells cannot depend on morphology and a limited set of neural markers. *Exp. Neurol.*
- Raedt R, Van Dycke A, Vonck K, Boon P, 2007. Cell therapy for refractory epilepsy. *Seizure-European Journal of Epilepsy.*
- Scharfman HE, Goodman JH, Sollas AL, 2000. Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. *J Neurosci* 20: 6144-6158.
- Semah F, Picot MC, Adam C, Broglin D, Arzimanoglou A, Bazin B, Cavalcanti D, Baulac M, 1998. Is the underlying cause of epilepsy a major prognostic factor for recurrence? *Neurology* 51: 1256-1262.
- Shapiro LA, Ribak CE, 2005. Integration of newly born dentate granule cells into adult brains: hypotheses based on normal and epileptic rodents. *Brain Res. Brain Res. Rev.* 48: 43-56.
- Smith PD, Mclean KJ, Murphy MA, Turnley AM, Cook MJ, 2005. Seizures, not hippocampal neuronal death, provoke neurogenesis in a mouse rapid electrical amygdala kindling model of seizures. *Neuroscience* 136: 405-415.
- Spencer SS, 2002. When should temporal-lobe epilepsy be treated surgically? *The Lancet Neurology* 1: 375-382.
- The Vagus Nerve Stimulation Group, 1995. A randomized controlled trial of chronic vagus nerve stimulation for treatment of medically intractable seizures. *Neurology* 45: 224-230.
- Thompson K, Anantharam V, Behrstock S, Bongarzone E, Campagnoni A, Tobin AJ, 2000. Conditionally immortalized cell lines, engineered to produce and release GABA, modulate the development of behavioral seizures. *Exp Neurol* 161: 481-9.

Thompson KW, Suchomelova LM, 2004. Transplants of cells engineered to produce GABA suppress spontaneous seizures. *Epilepsia* 45: 4-12.

Thompson KW, 2005. Genetically engineered cells with regulatable GABA production can affect afterdischarges and behavioral seizures after transplantation into the dentate gyrus. *Neuroscience* 133: 1029-1037.

Turner DA, Shetty AK, 2003. Clinical prospects for neural grafting therapy for hippocampal lesions and epilepsy. *Neurosurgery* 52: 632-644.

Vonck K, Boon P, Achten E, De RJ, Caemaert J, 2002. Long-term amygdalohippocampal stimulation for refractory temporal lobe epilepsy. *Ann. Neurol.* 52: 556-565.

Vonck K, Thadani VY, Gilbert K, Dedeurwaerdere S, De Groote L, De Herdt V, Goossens L, Gossiaux F, Achten E, Thierry E, Vingerhoets G, van Roost D, Caemaert J, De Reuck J, Roberts D, Williamson P, Boon P, 2004. Vagus nerve stimulation for refractory epilepsy: A transatlantic experience. *Journal of Clinical Neurophysiology* 21: 283-289.

Wiebe S, Blume WT, Girvin JP, Eliasziw M, 2001. A randomized, controlled trial of surgery for temporal-lobe epilepsy. *N. Engl. J Med* 345: 311-318.

Yagita Y, Kitagawa K, Ohtsuki T, Takasawa K, Miyata T, Okano H, Hori M, Matsumoto M, 2001. Neurogenesis by progenitor cells in the ischemic adult rat hippocampus. *Stroke* 32: 1890-1896.

Chapter 5

Cell transplantation for TLE

MANUSCRIPT 3

*RAEDT R. PINXTEREN J., VAN DYCKE A., WAEYTENS A., CRAEYE D.,
TIMMERMANS F., VONCK K., VANDEKERCHOVE B., PLUM J., BOON P.*

Differentiation assays of bone marrow-derived Multipotent Adult Progenitor Cell (MAPC)-like cells towards neural cells cannot depend on morphology and a limited set of neural markers.

Experimental Neurology 203 (2007), 542-554.

Differentiation assays of bone marrow-derived Multipotent Adult Progenitor Cell (MAPC)-like cells towards neural cells cannot depend on morphology and a limited set of neural markers

Robrecht Raedt ^{a,*}, Jef Pinxteren ^b, Annelies Van Dycke ^a, Anouk Waeytens ^c, David Craeye ^d,
Frank Timmermans ^b, Kristl Vonck ^a, Bart Vandekerckhove ^{b,d}, Jean Plum ^b, Paul Boon ^a

^a *Laboratory for Clinical and Experimental Neurophysiology, Department of Neurology, Ghent University Hospital, Belgium*

^b *Department of Clinical Chemistry, Microbiology and Immunology, Ghent University Hospital, Belgium*

^c *Department of Pathology, Ghent University Hospital, Belgium*

^d *Flemish Blood Service, Red Cross Flanders, Ghent, Belgium*

Received 29 January 2006; revised 30 August 2006; accepted 18 September 2006

Available online 31 October 2006

In this manuscript we compared the neuropotency of bone marrow-derived multipotent stem cells with that of brain-derived NSC. Because we isolated and expanded the bone marrow-derived cells as described for MAPC but the cells did not display the same multilineage potential we termed those cells MAPC-like cells. Both the morphology and the expression of five neural antigens were compared between NSC and MAPC-like cells before and after neural differentiation by three different protocols. To compare neural antigen expression both real-time polymerase chain reaction (RT-PCR) and immunocytochemistry were used.

SUMMARY

There is accumulating evidence for a neurogenic potential of bone marrow -derived cells both *in vitro* as well as *in vivo*. Most claims of neural “transdifferentiation” have based their conclusions on morphology and neural gene expression. Recently, doubts have been raised about the validity of both outcome parameters since non-neural cells can extend neurites and show aberrant neural gene expression as a response to stress inducing factors. In this study we compared bone marrow-derived Multipotent Adult Progenitor Cell (MAPC)-like cells and neural stem cells (NSC) in their morphology and neural gene expression profile after neural differentiation using three differentiation protocols. We evaluated the expression of five neuro-glial antigens [neurofilament 200 (NF-200); beta III tubulin ($\beta 3$ tub); tau; glial fibrillary acidic protein (GFAP); myelin basic protein (MBP) and RIP antigen] using real time PCR (RT-PCR) and immunocytochemistry (ICC). MAPC-like cells adopted a neural-like morphology in one protocol but a fibroblast-like morphology in the two other protocols. RT-PCR and ICC show that MAPC-like cells already express the neural antigens beta III tubulin and NF200 at baseline, but without upregulation of these genes after exposure to three distinct differentiation protocols. In contrast, NSC adopt neural and glial morphologies with a clear increase in expression of all neuro-glial genes in all differentiation protocols used. In conclusion, our data demonstrate that neural-like morphology and expression of a limited set of neural marker genes by MAPC-like cells after differentiation is no absolute proof of neural transdifferentiation because MAPC-like cells only partially meet the criteria which are fulfilled by NSC after neural differentiation.

INTRODUCTION

An adult stem cell source that is easy accessible and can be differentiated towards different neural cell types could be the ultimate cell source for cell therapy of neurodegenerative diseases. It is therefore not surprising that initial reports of a possible neurogenic potential of bone marrow-derived cells led to an explosion of reports which claimed that bone marrow-derived cells can adopt a neural identity (Eglitis and Mezey, 1997; Brazelton and others, 2000; Mezey and others, 2000; Priller and others, 2001; Nakano and others, 2001; Zhao and others, 2002; Mezey and others, 2003; Weimann and others, 2003; Munoz-Elias and others, 2004). However, more recent studies have clearly shown that such *in vivo* differentiation may be partial, extremely rare (Wagers and others, 2002) or may be artifactual (Alvarez-Dolado and others, 2003). In some studies, donor-derived cells are found in the brain after transplantation but these do not express neural antigens (Castro and others, 2002; Massengale and others, 2005). In other studies a fraction of the transplanted bone marrow cells do express neural antigens but these cells have a morphology distinct from adult neurons (Brazelton and others, 2000; Mezey and others, 2000; Zhao and others, 2002). Studies in which differentiation from blood stem cells to neurons was quantified, measured an extremely low efficiency of differentiation towards neural cells (Wagers and others, 2002). Original reports that bone marrow cells could differentiate towards Purkinje neurons were challenged by others, reporting that these cells were the result of fusion of donor cells with endogenous Purkinje neurons (Alvarez-Dolado and others, 2003; Weimann and others, 2003).

Next to these *in vivo* studies, several *in vitro* studies have shown that stem cells could be isolated out of bone marrow and that these stem cells were capable of transdifferentiation towards neural-like cells (Sanchez-Ramos and others, 2000; Woodbury and others, 2000; Deng and others, 2001; Kohyama and others, 2001; Kim and others, 2002; Jiang and others, 2002; Kabos and others, 2002; Rismanchi and others, 2003; Jiang and others, 2003; Levy and others, 2003; Locatelli and others, 2003; Padovan and others, 2003; Dezawa and others, 2004; Munoz-Elias and others, 2004; Tondreau and others, 2004; Magaki and others, 2005; Bossolasco and others, 2005). In some of these studies, bone marrow-derived cells were even capable of differentiation towards cells of the three different germinal layers (endoderm, mesoderm and ectoderm). These cells were called Multipotent Adult Progenitor Cells (MAPC) (Reyes and others, 2001; Jiang and others, 2002; Schwartz and others, 2002) or Marrow-Isolated Adult Multilineage Inducible (MIAMI) cells (D'Ippolito and others, 2004).

Most differentiation studies base their conclusions on morphological data, namely the formation of neurite like extensions, and immunohistochemical data, namely the expression of neural antigens, data. However recent evidence suggests that a neural-like morphology or neural antigen expression may not be as specific as previously thought. For example, Woodbury *et al.* recently developed a differentiation protocol where bone marrow stromal cells rapidly (within several hours) display neural morphology and show an enhanced expression of neural antigens by exposure to relatively simple chemicals such as dimethylsulfoxide (DMSO) and butylated hydroxyanisole (BHA) (Woodbury and others, 2000). However, further investigation with time-lapse video recording shows that the formation of neurites in this differentiation protocol is not the result of an

outgrowth of dendrite- and axon-like structures but rather a consequence of cell shrinkage and retraction of the cell edge (Neuhuber and others, 2004; Lu and others, 2004; Bertani and others, 2005). These “shrink-effects” are probably the consequence of stress on the cells caused by the chemicals used in the differentiation protocols. This assumption is based on the fact that exposure of the stromal cells, but also of other cell types (HEK 293 cells, fibroblasts), to different stressors (actin filament-depolymerising agents, detergents, high molarity sodium chloride, extremes of pH ...) results in the same type of morphology change. These results are strongly in favour of the hypothesis that the “neural morphology” of bone marrow-derived cells using the Woodbury protocol is in fact a “stress”- artefact.

Similarly, the relevance of an enhanced expression of neural antigens as an indication for neural differentiation should also be questioned for several reasons. First, some neural antigens are already expressed at baseline in native, non-neural bone marrow-derived cells (Woodbury and others, 2002; Goolsby and others, 2003; Neuhuber and others, 2004; Tondreau and others, 2004; Ratajczak and others, 2004; Bertani and others, 2005). Second, the exposure of mesenchymal stem cells to stressors also cause an enhancement of the expression of neural markers Neuronal Nuclei (NeuN) and Neuron Specific Enolase (NSE) (Lu and others, 2004), but also Neurofilament 200 (NF200) and Tau (Bertani and others, 2005). Finally, when different studies using the same type of stem cells and the same differentiation protocol are compared for antigen expression before and after differentiation, several inconsistencies can be found for different markers (Woodbury and others, 2000; Neuhuber and others, 2004; Lu and others, 2004; Bertani and others, 2005).

To better evaluate neural differentiation of bone marrow-derived cells, we compared neural differentiation of bone marrow-derived MAPC-like cells and brain-derived neural stem cells (NSC) using various differentiation protocols. We evaluated the expression of five different neuro-glial antigens (NF-200, beta III tubulin, Tau, Glial fibrillary acidic protein, myelin basic protein) at baseline and after differentiation. The expression profile of the neuroectodermal genes was measured using immunocytochemistry (ICC) and real-time PCR (RT-PCR). It was found that although bone marrow-derived cells can obtain some characteristics of neural cells, only neural stem cells gave rise to cells which met all criteria screened for.

MATERIALS AND METHODS

CULTURE OF MAPC-LIKE STEM CELLS

All animal procedures and experiments were approved by the local Ethical Committee of the University of Ghent. Isolation of bone marrow-derived stem cells was performed using the same procedure as described for MAPC (Jiang and others, 2002). In brief, the femurs of adult Sprague-Dawley rats (n=2, body weight 250 g, Harlan) were flushed and bone marrow mononucleated cells (BMMNC) were collected by Ficoll-Hypaque separation. BMMNC's were initially plated in expansion medium on fibronectin coated plates at a density of 6×10^5 cells/cm².

MAPC expansion medium consisted of the following: 57.5 % low glucose DMEM (Cambrex, Verviers, Belgium), 39 % MCDB-201 (Sigma, Bornem, Belgium), 1 % insulin-transferrin selenium (ITS, Sigma), 0.5 % linoleic acid-bovine serum albumin (LA-BSA, Sigma), 100 μ M ascorbic acid 2-phosphate (Sigma), 100U/ml penicillin (Cambrex), 100U/ml streptomycin (Cambrex), 10 ng/ml PDGF-BB (R&D), 10ng/ml EGF (Sigma), 2 % Fetal Calf Serum (FCS, Serum Supreme, Cambrex) and 1000 units/mL mLIF (Chemicon, Temecula, CA, USA). Half of the medium was changed every three days. After three weeks, the hematopoietic fraction (CD45⁺ and red blood cells) were depleted using paramagnetic beads. Cells were separated with 2 sequential MACS LD columns (Miltenyi Biotech, Utrecht, The Netherlands). After depletion, cells were plated in fibronectin coated flat-bottomed 96 well plates at a density of 1 cell per well to generate single cell clones. Clones were expanded at densities between 0.5 and 1.5×10^3 /cm².

Five single cell MAPC-like clones were withdrawn on the basis of their spindle-like morphology, their expansion capacity (> 40 population doublings), and their phenotypic expression profile (MHC I⁻, MHC II⁻, CD44^{-dim}) using flow cytometry analysis. Using Real-Time Polymerase Chain Reaction (RT-PCR) relative expression of Oct4 was compared between the five MAPC-like cell clones and the clone with the highest expression was chosen to be used for further analysis in this study.

CULTURE OF NEURAL STEM CELLS

Neural stem cells were isolated according to a recently published protocol (Gobbel and others, 2003). In brief, the brain of female Sprague-Dawley rats (n=2, body weight 250 g, Harlan) was removed and placed in artificial cerebrospinal fluid (124 mM NaCl, 5 mM KCl, 3.2 mM MgCl₂, 105 mM NaHCO₃, 10 mM glucose, and 2 mM CaCl₂). The subventricular zone was removed bilaterally by microscopic dissection. To dissociate the tissue, it was placed into a digestion medium containing 0,5 mM Na₂EDTA (Sigma), 1,0 mM cysteine (Sigma), 0,9 mg/ml papain (Worthington, Lakewood, NJ) and 1 mg/ml DNase (Sigma), dissolved in Earle's balanced salt solution and this for 45 minutes. Digestion was stopped by adding ovomucoid (Sigma). After a mechanical dissociation, the cells were plated in a 96 well flat-bottomed plate at a density of 2000 cells per well in a chemically defined medium. The NSC growth medium consisted of NS-A medium (Euroclone, Milan, Italy) with an additional 2 mM

L-glutamine (Cambrex), 3 mM D-glucose (Sigma), 1.2 mM sodium bicarbonate (Sigma), 0.46 mM HEPES (Sigma), 2 % B27 (Gibco BRL, Rockville, MD), 1 % N2-supplement (Gibco BRL), 100 U/ml penicillin (Cambrex), 100 U/ml streptomycin (Cambrex), 20 ng/ml of human recombinant epidermal growth factor (EGF; Sigma), and 20 ng/ml of human recombinant basic fibroblast growth factor (bFGF; R&D, Minneapolis, MN, USA). Cells were grown at 37 °C in 5 % CO₂ and 95 % air with saturated humidity. They were passaged once cell clusters were formed, approximately 2 weeks after initial isolation and every 1–2 weeks thereafter. At the fourth passage, the cells were plated in a flat-bottomed 96 well plate at a density of 1 cell per well. A single cell clone was chosen to be used in this study on the basis of its capacity to form neurospheres and the survival and morphology after *in vitro* differentiation.

KARYOTYPING

MAPC-like cells and NSC, subcultured at a 1:4 dilution 24 hours before harvesting, were collected and subjected to a 1.5-hour colcemid incubation, followed by lysis with hypotonic KCL and fixation in acid/alcohol. Metaphases were analyzed after G banding (Dewald and others, 1985).

DIFFERENTIATION OF MAPC-LIKE CELLS INTO MESENCHYMAL LINEAGES

ADIPOGENESIS

To induce MAPC-like cells to differentiate into adipocytes, cells were plated into six-well plates at a density of $20 \times 10^3/\text{cm}^2$ and incubated in adipogenic induction medium consisting of DMEM supplemented with 10 % FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 1 µM dexamethasone, 0,2 mM indomethacin, 10 µg/ml insulin, 0,5 mM methylisobutylxanthine (all from Sigma). Medium was replaced every 3-4 days during 3 weeks. Differentiation was appreciated by morphological examination and Oil Red O staining was performed to detect lipid accumulation (Pittenger and others, 1999).

OSTEOGENESIS

To induce MAPC-like cells to differentiate into osteoblasts, cells were plated into six-well plates at a density of $3 \times 10^3/\text{cm}^2$ and incubated in osteogenic induction medium consisting of DMEM supplemented with 10 % FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 0,1 µM dexamethasone, 50 µM ascorbic acid 2-phosphate, 10 mM beta-glycerophosphate (Sigma). Medium was replaced every 3-4 days during 3 weeks. Von Kossa's staining was performed in order to demonstrate calcium deposition in differentiated cultures (Pittenger and others, 1999).

NEURAL DIFFERENTIATION OF MAPC-LIKE CELLS AND NSC

All differentiations were done with the cells plated on matrigel in DMEM/B27 medium (Gibco BRL) with an additional 2 mM L-glutamine (Cambrex), 3 penicillin mM D-glucose (Sigma), 1,2 mM sodium bicarbonate (Sigma), 0,46 mM HEPES (Sigma), 2 % B27 (Gibco BRL), 1 % N2-supplement (Gibco BRL), 100U/ml (Cambrex),

100U/ml streptomycin (Cambrex). In order to enhance survival of NSC during differentiation 3 % FCS was added to the differentiation medium. MAPC-like cells were differentiated in medium devoid of FCS in order to induce differentiation. Cells were differentiated parallel by three methods based on previously published protocols: the Jiang method (Jiang and others, 2003), the Kohyama method (Kohyama and others, 2001) and the Kabos method (Kabos and others, 2002). In brief, for MAPC-like cells, the Jiang method consisted of a predifferentiation phase in which the cells are exposed to bFGF (100 ng/ml, R&D) for seven days. After this neural induction phase MAPC-like cells were exposed to Sonic Hedgehog (100 ng/ml, R&D) and FGF-8 (10 ng/ml, R&D) for another seven days. In the case of NSC differentiation the cells were directly plated for seven days in the presence of SHH (100 ng/ml) and FGF-8 (10 ng/ml) without a predifferentiation phase. In the Kohyama protocol MSC cells were incubated for three days with 10 µmol/ml 5-azacytidine, 10 % FCS, Nerve Growth Factor (NGF, R&D, 50 ng/ml), Brain Derived Factor (BDNF, R&D, 50 ng/ml) and Neurotrophin-3 (NT-3, R&D, 50 ng/ml) for the induction of neural differentiation. After the induction phase, cells were incubated with serum-free medium containing NGF, NT-3, BDNF (all 50 ng/ml) for seven days. NSC cells were not subjected to the induction phase and were directly plated for seven days in the presence of the cytokines NGF, NT-3 and BDNF (all 50 ng/ml). In the Kabos method cells were incubated for seven days with 20 ng/ml bFGF and 20 ng/ml EGF in order to induce differentiation. In the following phase, cells were exposed to 1 µM retinoic acid (RA, sigma) and 1mM dibutyl cyclic AMP (db-cAMP, sigma) for an additional seven days. NSC were directly plated in medium containing 1 µM RA and 1 mM db-cAMP and this also for seven days.

After seven days of differentiation, morphology of the cells was evaluated, a fraction of the cells were fixated for immunocytochemistry and RNA was extracted from the remaining cell fraction.

REAL-TIME POLYMERASE CHAIN REACTION FOR OCT4 AND NEURO-GLIAL MRNA'S

RNA was extracted from the cells using a kit (Aurum™ total RNA kit, Bio-Rad, Hercules, CA, USA). cDNA was prepared using a first strand synthesis kit (iScript™ cDNA Synthesis Kit, Bio-Rad). Using the standard curve method the relative amount of expression of Oct4 and neuroectodermal genes was determined by normalization using hypoxanthine phosphoribosyl transferase (HPRT) as a housekeeping gene (Peinnequin and others, 2004). For HPRT, the oligonucleotides used were GCG TCC CTT TTG ATT TGC A (sense primer) and TCA CAA GGG AAG TGA CAA TCT ACC T (antisense primer), for Oct4 CTG TAA CCG GCG CCA GAA (sense primer) and TGC ATG GGA GAG CCC AGA (antisense primer), for beta3-tubulin (β3-tub) GGA GCT GTT CAA GCG CAT CT (sense primer) and CCC GTG TAC CAG TGG AGG AA (antisense primer), for NF200 GCTG TCC TTC TTG AGC TTC TTT G (sense primer) and TTG TTG TCG GGC TAT GAA GAA A (anti-sense primer), for Tau AAG CCA GTG GAC CTG AGC AA (sense primer) and TTC GAC TGG ACT CTA TCC TTG AAG T (antisense primer), for Glial Fibrillary Acidic Protein (GFAP) GAG AGA TTC GCA CTC AGT ACG A (sense primer) and TGG ACC GAT ACC ACT CTT CTG TT (antisense primer), for Myelin Basic Protein (MBP) ATT CCG AGG AGA GTG TGG GTT T (sense primer) and GAA GTT TCG TCC CTG CGT TTC (antisense primer). All primers were designed with Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) and tested for specificity using NCBI's BLAST software. All primers were manufactured and HPLC purified by Operon Biotechnologies (Cologne, Germany). PCR reagents were

obtained from Eurogentec (Seraing, Belgium) as SYBR Green I mastermixes and used according to the manufacturer's instructions. Reactions were run on ABI Prism 7000 Sequence Detection System (Applied Biosystems). The cycling conditions were as follows: 10 min at 95°C, 40 cycles at 95°C for 15 s and 60°C for 60 s. After PCR amplification, a melting curve was generated for every PCR product to check the specificity of the PCR reaction (absence of primer-dimers or other non-specific amplification products). To quantify gene expression, normalized mRNA expression levels of Oct4 in the five MAPC-like cells clones were compared with levels in rat testis. Normalized mRNA expression levels of neuro-glial genes in MAPC-like cells and NSC before (respectively M0 and N0) and after differentiation according to the Jiang (M1, N1); the Kohyama (M2, N2) and the Kabos (M3, N3) protocol were compared with levels of expression in a NSC clone that was differentiated following a standard protocol (N_{standard}). According to this protocol, cells are plated on poly-D-lysine in medium devoid of growth factors but with the addition of 3 % Fetal Bovine Serum (Reynolds and Weiss, 1996; Gobbel and others, 2003). Therefore, the formula to quantify expression of the genes of interest ($\text{Genes}_{\text{int}}$) is: $[\text{Relative expression (Gene}_{\text{int}}) \text{ in } N_x \text{ or } M_x] / [\text{Relative expression (Gene}_{\text{int}}) \text{ in } N_{\text{standard}} / \text{Relative expression (HPRT) in } N_{\text{standard}}]$.

IMMUNOCYTOCHEMISTRY FOR CNS ANTIGENS

Undifferentiated and differentiated NSC and MAPC-like cells were stained for the same markers from which the gene expression was analysed using RT-PCR, except for oligodendrocytes, which were stained with RIP antibody because this resulted in stainings compared to immunocytochemistry for MBP. Cells were fixated with 4 % paraformaldehyde for 10 minutes and subjected to immunocytochemistry. MAPC-like cells and differentiated NSC are adherent and can be processed easily. In order to prevent differentiation induced by plating undifferentiated NSC on coated surface, cytocentrifuged preparations (Cytospin 2; Cytospin, Shandon, UK) were made and fixated. The PFA-fixed preparations were quenched with 50 mM NH_4Cl for 10 min, permeabilized with 0.2–0.5 % Triton X-100 (TX100) in phosphate-buffered saline (PBS) for 5 min, and blocked using 0.4 % fish skin gelatin/PBS for 30 min, followed by incubation first with primary antibodies (ms, mouse monoclonal; rb, rabbit polyclonal): ms anti-nestin 1:50 (chemicon, MAB 353), ms anti- β 3 tub 1:100 (Sigma SDL-SD10), ms anti-NF200 1:100 (chemicon, MAB5262), rb anti-tau 1:5000 (Dako, A 0024), rb anti-GFAP, 1:100 (Dako, Z0334), ms anti-oligodendrocytes 1:5000 (Chemicon, RIP, MAB1580) and then with secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes) 1:1000 and goat anti-rabbit Texas Red IgG (H+L) (Vector Laboratories, Burlingame, CA) 1:1000 diluted in 0.4 % fish skin gelatin/phosphate-buffered saline for 2 h each. To test the specificity of the secondary antibodies the primary antibody step was omitted. Cross reactivity of the primary antibodies with human antigens allowed testing the antigen specificity by staining HUVEC cells as a negative control and rat brain as a positive control. The coverslips were mounted in Mowiol supplemented with 4,6-diamidino-2-phenylindole (DAPI). Immunoreactive (IR) cells were visualized using a Bio-Rad Radiance 2100 confocal laser scanning microscope.

RESULTS

ISOLATION OF MAPC-LIKE CELLS

We successfully isolated cells from the bone marrow of adult rats using the protocol described by Jiang *et al.* (Jiang and others, 2002). After three weeks, bulk cultures were depleted of CD45⁺ and red blood cells and were subsequently plated in MAPC expansion medium at a density of one cell per well. Five clones were selected on the basis of a similar morphology (fig 1), flowcytometric data (MHC I and MHCII neg, CD44^{-dim}) and growth characteristics (data not shown) as true MAPC cells. All these five clones were subjected to the neural differentiation protocol described in the study by Jiang *et al* (Jiang and others, 2002). Since none of the clones differentiated towards cells positive for tau, GFAP or RIP using this protocol, we will designate these cells as MAPC-like cells. After these initial differentiation attempts, we used RT-PCR analysis to determine the levels of Oct4 expression in these five cell clones (table 1). The clone with the highest expression, clone 3.5b1, was chosen to be used for further analysis.

Cytogenetic analysis after 40 population doublings showed that all clones had a normal karyotype (fig 2).

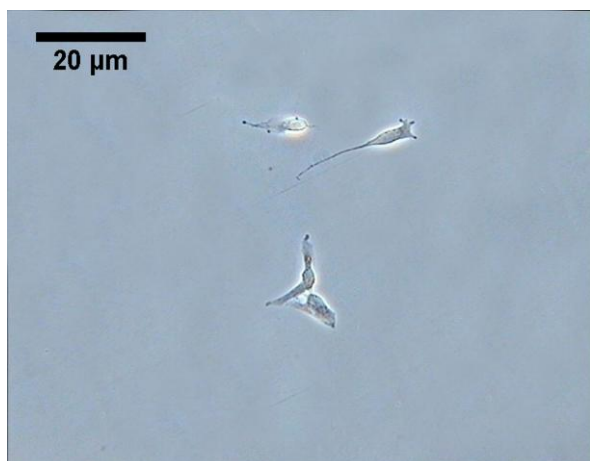


Fig 1: Morphology of bone marrow derived MAPC-like cells. Selection of MAPC-like cell clones was done on the basis of a high expansion capacity (> 40 population doublings) and a spindle like morphology. Figure 1 is a picture taken by a digital camera through the ocular of a stereomicroscope. The magnification of the stereomicroscope is set at 200x. On the picture the spindle like morphology of the MAPC-like cells clone is clear.

Table 1: Levels of Oct4 mRNA expression in five MAPC-like cell clones relative to expression in rat testis.

Clone	Oct4 expression in single cell clones relative to rat testes
RY	1.26±0.12
RS	0.88±0.35
3.5b2	0.18±0.05
3.4b5	0.28±0.09
3.5b1	1.89±0.16

RNA was extracted from five MAPC-like cell clones. cDNA was prepared using a first strand synthesis kit (iScriptTM cDNA Synthesis Kit, Bio-Rad). RT-PCR reactions were run on ABI Prism 7000 Sequence Detection System. The cycling conditions were as follows: 10 min at 95°C, 40 cycles at 95°C for 15 s and 60°C for 60 s. After PCR amplification, a melting curve was generated for every PCR product to check the specificity of the PCR reaction. Using the standard curve method the relative amount of expression of Oct4 was determined by normalization using HPRT as a housekeeping gene. To quantify gene expression, normalized mRNA expression levels of Oct4 in MAPC-like cell clones were compared with levels of expression in rat testes.

ISOLATION AND PHENOTYPIC CHARACTERISATION OF NSC CELLS

Neural stem cells were isolated from the subventricular zone of the brain by plating dissociated, subventricular cells in a special defined neural stem cell expansion medium (Gobbel and others, 2003). Two weeks after initial isolation, large spherical clusters of cells, or neurospheres, which grew loosely attached to the substrate, were present in the culture. These neurospheres were passaged mechanically when the diameter of the neurospheres ranged from 100 to 150 μm . The dissociated cells were plated again and secondary neurospheres appeared in culture. Neurospheres were passaged every 7 to 10 days after plating. At the fourth passage, the cells were plated at a density of 1 cell per well in a 96 well plate. Approximately 4 % of the cells formed neurospheres which could be passaged continuously (>10 passages). Using the culture and isolation technique specially designed for adult rat neural stem cells, we were able to keep single cell clones of NSC in culture for over one year (Gobbel and others, 2003). Formation of neurospheres and expansion capacity were used as criteria for the selection of a clone which was used in this study. Cytogenetic analysis of the cells after 40 population doublings showed that cells also had a normal karyotype (data not shown).

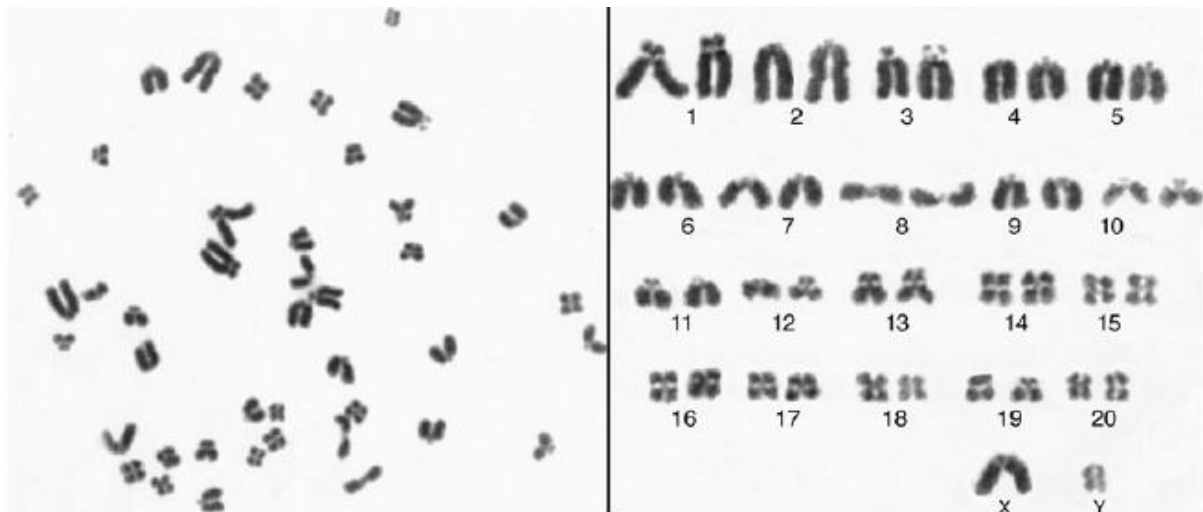


Fig 2: Representative example of a metaphase spread (left) and an ideogram (right) of G banded karyotype of MAPC-like cells. MAPC-like cells were passaged for 40 cell doublings. Cells were harvested 24 hours after replating, subjected to a 1.5-hour colcemid incubation followed by lysis with hypotonic KCL and fixation in acid/alcohol and cytogenetically analyzed. Metaphase spread and ideogram show no large chromosomal abnormalities. Karyotype analysis of NSC gave analogue results (data not shown).

MESENCHYMAL DIFFERENTIATION OF MAPC-LIKE CELLS

When inducing the adipogenic differentiation, the cells adopted a round morphology and accumulated large, cytoplasmic vacuoles in which accumulation of lipid was detected with Oil Red O staining (fig 3a), while in non-induced cells no staining was detected (fig 3b). The osteogenic differentiation was demonstrated by calcium deposition in cell culture by positive Von Kossa's stain in large portion of cells (fig 3c) while non-induced cells were all negative (fig 3d).

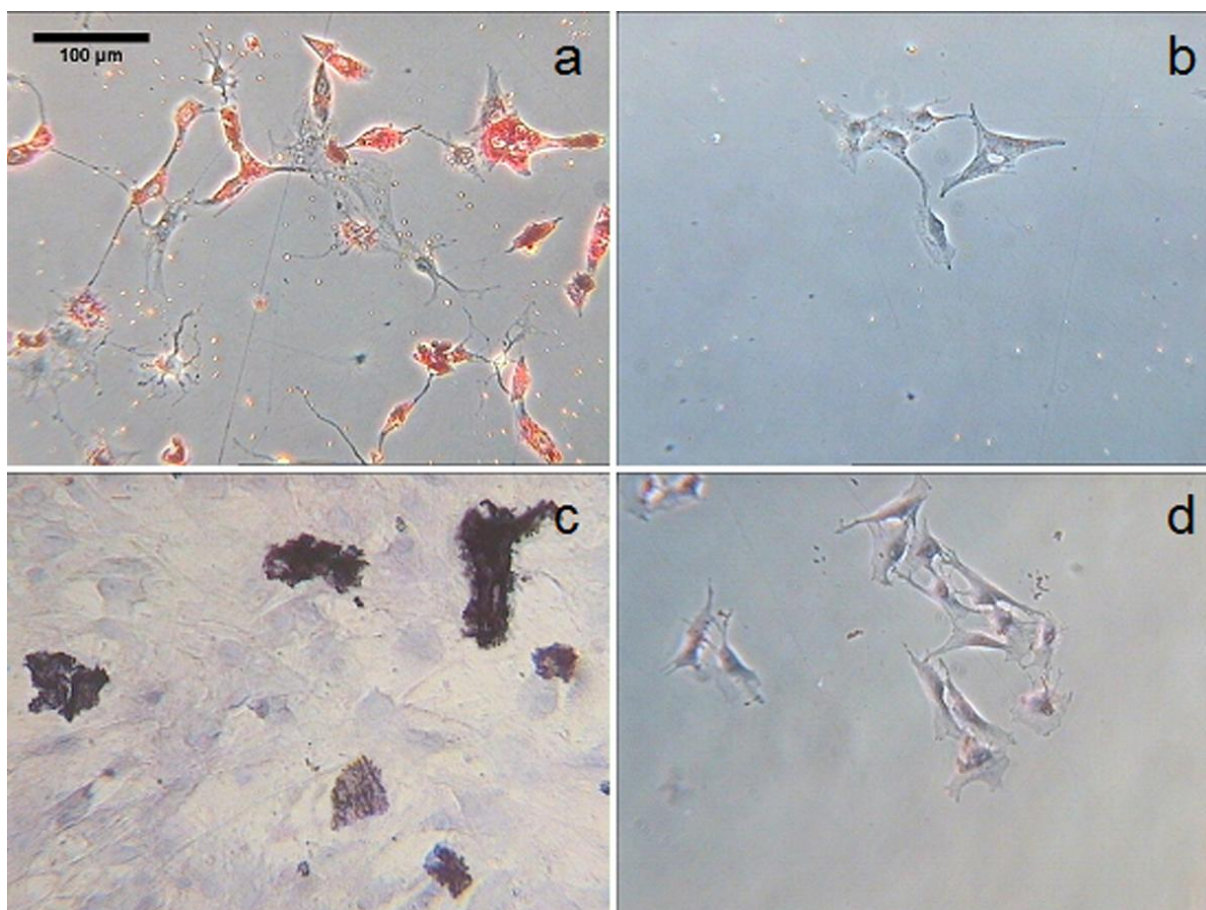


Fig 3: Mesenchymal differentiation of MAPC-like cells. a and b: Adipogenic differentiation was induced by plating MAPC-like cells at a density of $20 \times 10^3/\text{cm}^2$ in the presence of 10 % FCS, 1 μM dexamethasone, 0,2 mM indomethacin, 10 $\mu\text{g}/\text{ml}$ insulin, 0,5 mM methylisobutylxanthine. The cells adopted a round morphology and accumulated large, cytoplasmic vacuoles in which accumulation of lipid was detected with Oil Red O staining (a), while in non-induced cells no staining was detected (b). c and d: Osteogenic differentiation was induced by plating MAPC-like cells at a density of $3 \times 10^3/\text{cm}^2$ in the presence of 10 % FCS, 0,1 μM dexamethasone, 50 μM ascorbic acid 2-phosphate, 10 mM beta-glycerophosphate. In these osteoblasts calcium deposition could be demonstrated by Von Kossa's staining (c) while non-induced cells were all negative (d).

NEURAL DIFFERENTIATION OF MAPC-LIKE CELLS AND NSC

MAPC-like cells and NSC were differentiated according to three previously described protocols: the Jiang method (Jiang and others, 2003), the Kohyama method (Kohyama and others, 2001) or the Kabos method (Kabos and others, 2002). In contrast to NSC, MAPC-like cells were first predifferentiated by incubation with neural induction factors: bFGF (Jiang method); 5-azacytidine/NGF/NT-3/BDNF (Kohyama method) and bFGF/EGF (Kabos method). In all three methods, MAPC-like cells formed neurite like extensions and in case of the Jiang and Kabos method the cells rounded up and appeared as very bright cells by phase contrast microscopy (fig 4a). Quantification however showed that the proportion of cells that immunostain positive for nestin, a marker for neuroprogenitor cells, was lower after predifferentiation (about 15%) than at baseline conditions (about 30%, fig 4b).

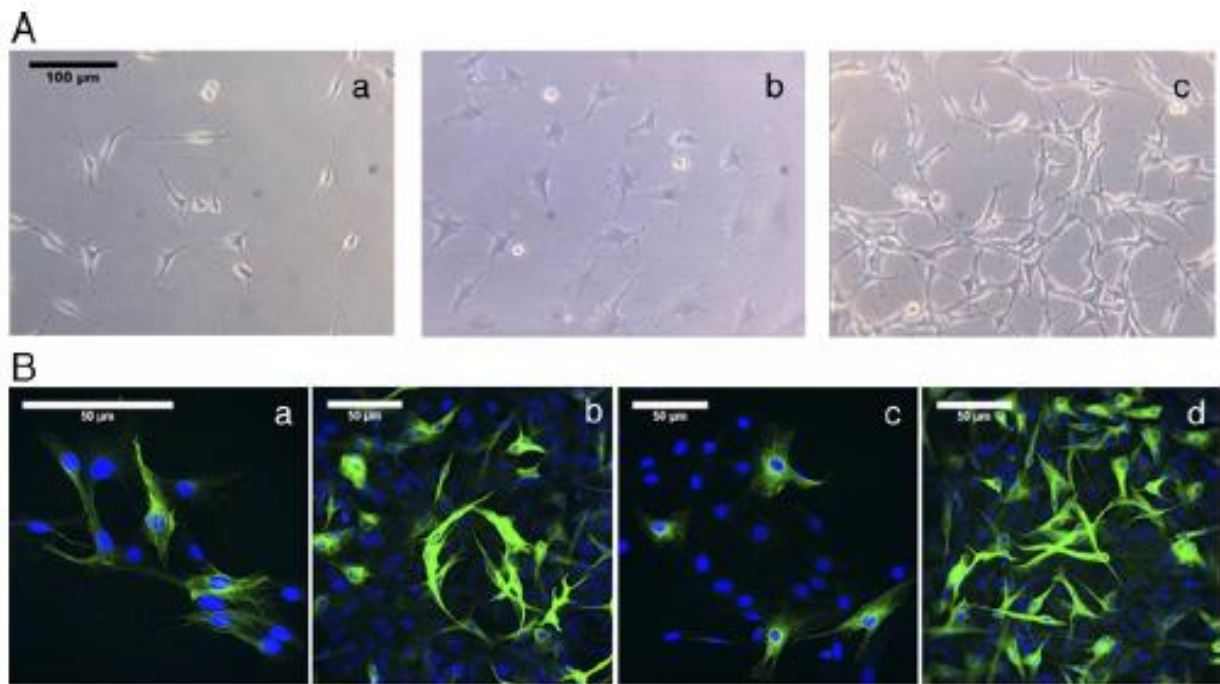


Fig 4: Comparative analysis of morphology and expression of nestin on predifferentiated MAPC-like cells towards neural cells using 3 different protocols. A: Neural predifferentiation of MAPC-like cells by plating them on matrigel in the presence of (a, Jiang method) 100 ng/ml bFGF; (b, Kohyama method) 5-azacytidine (10 μ mol/ml), NGF (50 ng/ml), NT-3 (50 ng/ml), BDNF (50 ng/ml), 10 % FBS; (c, Kabos method) EGF (20 ng/ml) and bFGF (20 ng/ml). In all three cases cells extend processes and in the presence of bFGF (a and c) the cells keep dividing, round up were very bright under phase contrast microscope view. B: Immunocytochemistry analysis showed that there is a clear increase in the number of cells expressing nestin in the Jiang (b) and the Kabos (d) differentiation method at the end of the predifferentiation phase compared to baseline condition where the MAPC-like cells are undifferentiated (a). The increase in nestin expression was not seen in the Kohyama differentiation (c).

Predifferentiated MAPC-like cells and NSC were then differentiated by culturing them for seven days in the presence of other differentiation factors (Jiang method: SHH, FG-8; Kohyama method: NGF, BDNF, NT-3; Kabos method: RA, cAMP). MAPC-like cells differentiated with the Jiang method displayed a bipolar morphology and rounded cell bodies, which is typical for young neurons. MAPC-like cells, differentiated with the Kohyama or Kabos method, also developed long extensions but adopted a more fibroblast-like morphology (fig 5). NSC cells displayed a large number of neurite-like processes in all three conditions. Cells with a bipolar morphology and cells with a polarized morphology were present. In addition, a significant fraction of cells with glial-like morphology could be detected between the differentiated cells.

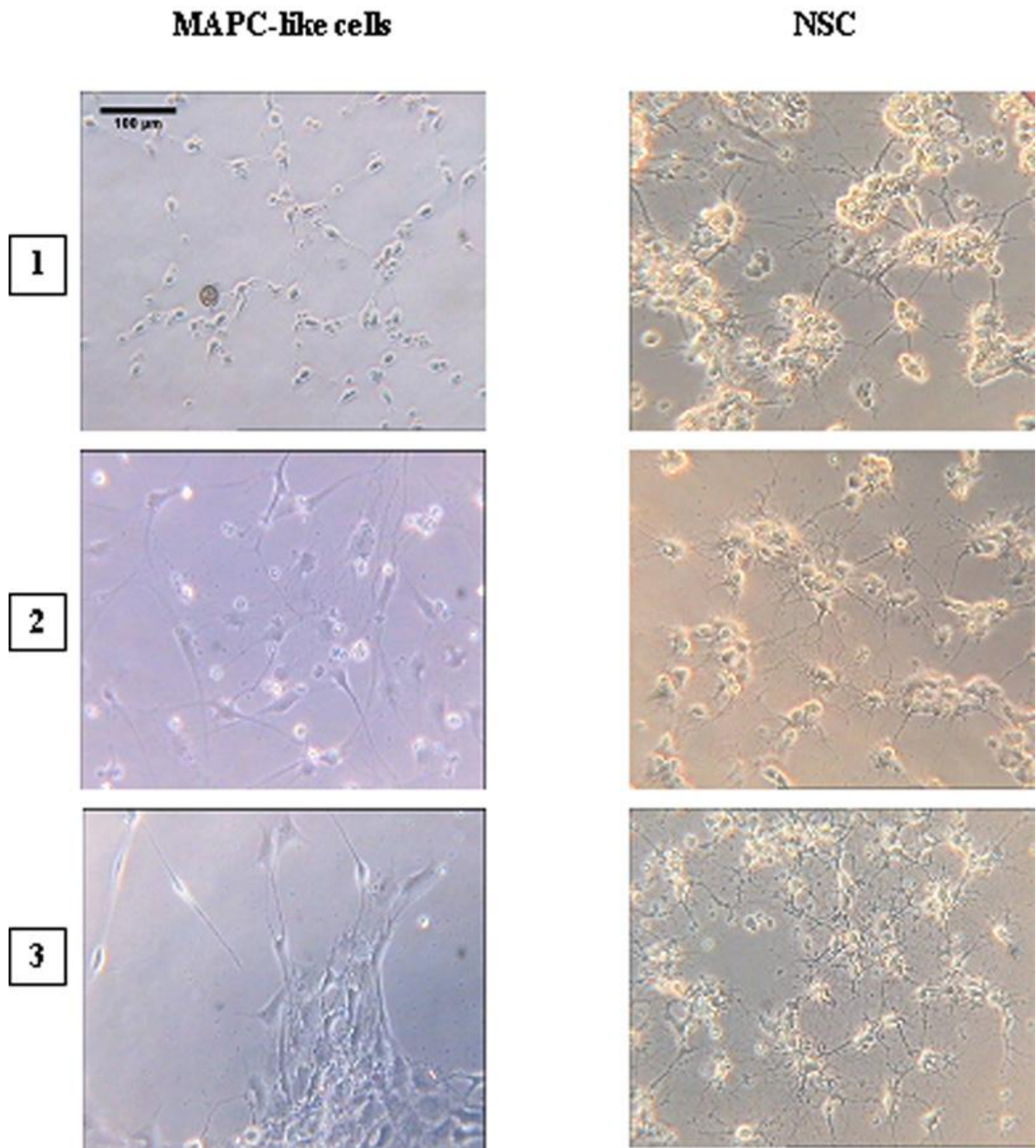


Fig 5: Comparative analysis of morphology of MAPC-like cells and NSC at the end of differentiation using 3 different protocols. At the end of differentiation morphology of the cells was evaluated. MAPC -like cells differentiated according to the Jiang protocol (M1) displayed bipolar morphology and round cell bodies, both typical for young neurons. Kohyama (M2) and Kabos (M3) differentiation led to cells displaying a more fibroblast-like morphology. NSC cells differentiated according to the N1, N2, N3 protocol all extended much neurite-like processes and cells with bipolar, polarized and glial morphology were present between the differentiated cells.

QUANTITATIVE REAL-TIME PCR FOR NEURO-GLIAL MRNA'S

Normalized mRNA expression levels of neuro-glial genes in MAPC-like cells and NSC before (respectively M0 and N0) and after differentiation according to the Jiang (M1, N1); the Kohyama (M2, N2) and the Kabos (M3, N3) protocol were calculated. The results demonstrate that neural markers β 3-tub and NF-200 are expressed in NSC before (N0) and after N1, N2 and N3 differentiation. Expression is however less than in the NSC clone,

differentiated using the standard protocol. In addition, the increase of β 3-tub and NF-200 expression after differentiation is limited: β 3-tub expression is 1.5 to 2 times higher after differentiation, NF200 expression is 7 to 8 times higher after differentiation. RT-PCR analysis showed that β 3-tub and NF-200 were also expressed in MAPC-like cells. β 3-tub expression was even higher in MAPC-like cells before (M0) than after differentiation (M1, M2, M3). In the case of NF200, expression in M0 was lower than M1 and M2 but higher than M3 expression. We subsequently analyzed expression of differentiation markers for neurons (Tau), astrocytes (GFAP) and oligodendrocytes (MBP). Expression of Tau, GFAP and MBP could not be demonstrated in MAPC-like cells before or after differentiation. In contrast, all three markers showed a baseline expression in NSC before differentiation. After differentiation, increase in expression was noted: 1 tenfold increase for tau, a 100-fold increase for MBP and a 1000-fold increase for GFAP. For all three markers there is a clear augmentation in expression due to differentiation (table 2).

Table 2: Comparative analysis of mRNA expression for different proteins of MAPC-like cells and NSC before and after differentiation using 3 different protocols

	beta III tubulin	NF200	Tau	GFAP	MBP
M0	0,121 \pm 0,002	0,033 \pm 0,002	0	0	0
M1	0,053 \pm 0,008	0,074 \pm 0,008	0	0	0
M2	0,051 \pm 0,001	0,057 \pm 0,001	0	0	0
M3	0,012 \pm 0,001	0,009 \pm 0,001	0	0	0
N0	0,427 \pm 0,056	0,127 \pm 0,056	0,561 \pm 0,042	0,005 \pm 0,001	0,005 \pm 0,000
N1	0,655 \pm 0,028	0,842 \pm 0,028	6,829 \pm 0,804	13,868 \pm 0,760	0,656 \pm 0,041
N2	0,743 \pm 0,085	0,970 \pm 0,085	7,744 \pm 0,524	18,549 \pm 1,340	0,782 \pm 0,042
N3	0,861 \pm 0,158	0,844 \pm 0,158	4,874 \pm 1,352	12,891 \pm 3,231	0,311 \pm 0,040

RNA was extracted from MAPC-like cells and NSC before (respectively M0 and N0) and after differentiation with the Jiang (M1, N1); the Kohyama (M2, N2) and the Kabos method (M3, N3). cDNA was prepared using a first strand synthesis kit (iScriptTM cDNA Synthesis Kit, Bio-Rad). RT-PCR reactions were run on ABI Prism 7000 Sequence Detection System. The cycling conditions were as follows: 10 min at 95°C, 40 cycles at 95°C for 15 s and 60°C for 60 s. After PCR amplification, a melting curve was generated for every PCR product to check the specificity of the PCR reaction. Using the standard curve method the relative amount of expression of neuroectodermal genes was determined by normalization using HPRT as a housekeeping gene. To quantify gene expression, normalized mRNA expression levels of neuro-glial genes in MAPC-like cells NSC before (respectively M0 and N0) and after differentiation according to the Jiang (M1, N1); the Kohyama (M2, N2) and the Kabos (M3, N3) protocol were compared with levels of expression in a NSC clone that was differentiated following a standard protocol (N_{standard}). According to this protocol cells are plated on poly-D-lysine in medium devoid of growth factors but with the addition of 3% Fetal Bovine Serum (FBS). The formula to quantify expression of the genes of interest (Genes_{int}) is: [Relative expression (Gene_{int}) in N_x or M_x]/[Relative expression (HPRT) in N_x or M_x]/ [Relative expression (Gene_{int}) in N_{standard}]/[Relative expression (HPRT) in N_{standard}].

Immunocytochemistry of NSC and MAPC-like cells yielded the following results. The presence of β 3-tub and NF200 could not be demonstrated in non-differentiated NSC; but differentiated NSC stained positive for these markers. β 3-tub and NF200 could also be demonstrated in MAPC-like cells and this before and after differentiation (figure 6A).

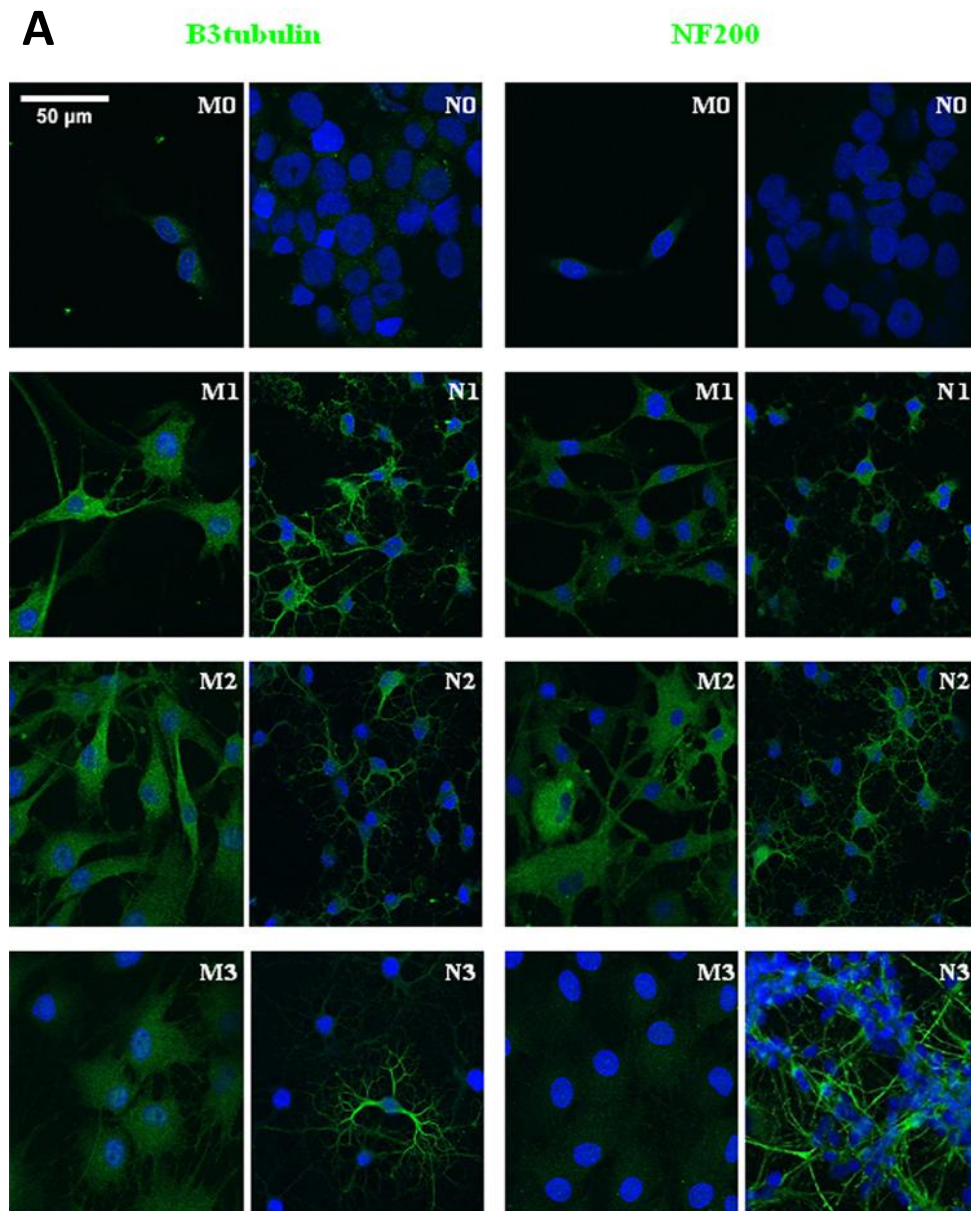
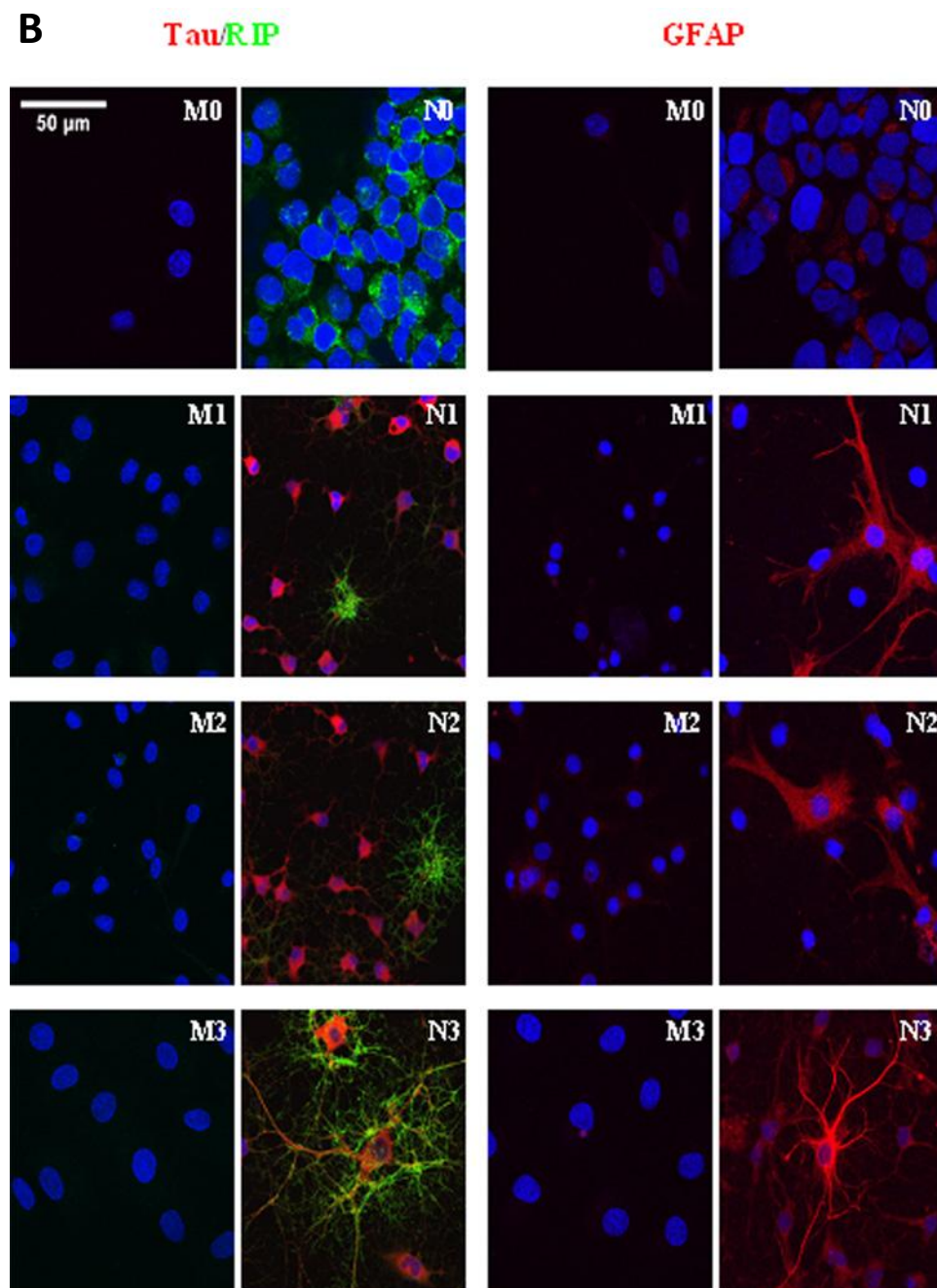


Fig 6: Comparative analysis of antigen reactivity of MAPC-like cells and NSC at the end of differentiation using 3 different protocols. Immunocytochemistry analysis of MAPC-like cells and NSC cells before (resp. M0 and N0) and after differentiation according to the Jiang protocol (M1, N1); the Kohyma method (M2, N2) and the Kabos method (M3, N3). A: M0 cells stained slightly positive for B3tub and NF200. N0 cells did not stain for NF200 and B3tub. Between the differentiated NSC and MAPC-like cells there were cells that stained positive for NF200 and B3tub. B: No TAU-, RIP- or GFAP-positive cells could be demonstrated either before or after differentiation. Undifferentiated NSC were negative for TAU and GFAP but some stained positive for the RIP marker. After all three differentiations TAU, GFAP and RIP positive cells were present among the differentiated cells.

Immunocytochemistry for Tau, RIP and GFAP showed that MAPC-like cells did not stain positive for these markers before or after differentiation in contrast to differentiated NSC. Undifferentiated NSC did only stain positive for RIP but not for TAU or GFAP (figure 6B). One can conclude from both techniques that β 3-tub and NF200 are expressed in MAPC-like cells both in undifferentiated as in differentiated state; whereas TAU, GFAP and MBP are not expressed in MAPC-like cells neither in undifferentiated nor in differentiated state. Both RT-PCR and ICC demonstrated that differentiated NSC were positive for all markers investigated and this in all three differentiation paradigms. RT-PCR could detect expression of β 3-tub, NF200, TAU and GFAP genes in undifferentiated neural stem cells while ICC could not detect the presence of these antigens.



DISCUSSION

Most studies that demonstrated neural differentiation of bone marrow-derived cells based their conclusion on the adoption of a neural-like morphology and the expression of neural genes. Recent evidence, however, shows that a non-neural cell can adopt a neural morphology and show aberrant expression of neural antigens as a response to different stressors rather than being the result of neural transdifferentiation (Neuhuber and others, 2004; Lu and others, 2004; Bertani and others, 2005). To further investigate the significance of neural morphology and neuroglial antigen expression as proof of neural differentiation, bone marrow-derived MAPC-like cells were compared in their morphology and neuro-glial gene expression profile with positive control NSC cells before and after neural differentiation by three different protocols.

In this study, we were able to isolate stem cells out of the mesenchymal cell fraction of rat bone marrow with an expansion capacity and a spindle-like morphology characteristic for MAPC cells, a rare subpopulation of MSC, described by Verfaillie et al. (Jiang and others, 2002). MAPC's have been shown to differentiate to cells of the three germinal layers (endoderm, ectoderm and mesoderm). We were able to differentiate the MAPC-like cells towards mesodermal cell fates, such as osteoblasts and adipocytes, using protocols described for mesenchymal stem cells. To date, no other group has published that pluripotent cells could be generated using the method described by Jiang in 2002 suggesting that the critical parameters necessary for terminal transdifferentiation are unknown. Pluripotency is a required property of MAPC but since we were also unable to reproducibly obtain differentiation to neuronal cells, we designate the cells MAPC-like. Because we were especially interested in the neural transdifferentiation capacity of bone marrow-derived MAPC-like cells, we compared the neural transdifferentiation capacity of a MAPC-like cell clone with that of tissue-specific NSC isolated from the subventricular zone of adult rats. Several studies have shown that NSC can differentiate towards electrically active neurons, astrocytes and oligodendrocytes (Whittemore and others, 1999; Toda and others, 2000; Liu and others, 2000; Song and others, 2002).

We selected a MAPC-like cell clone on the basis of its Oct4 expression which was highest compared to four other clones analyzed. Oct4 is also expressed in precursors of gametes or primordial germ cells (PGC), in undifferentiated embryonic stem cells (ESC), embryonic germ cells (EGC) and embryonic carcinoma cells (ECC). Its exact role is not yet elucidated, but expression of Oct4 in ESC is important in maintaining self-renewal and a pluripotent state (Niwa and others, 2000; Cavaleri and Schöler, 2006). This seems also to be the case in MAPC since clones with high Oct4 expression have a higher pluripotency than clones with low Oct4 expression (Verfaillie, Personal Communication).

NSC could be successfully isolated, cultured and differentiated using a standard protocol (Gobbel and others, 2003). The NSC were subjected to three differentiation protocols which were specifically designed for neuronal differentiation of bone marrow-derived stem cells. The protocols, however, were slightly modified taking into account the different nature of the cells. The most important modification was the omission of the neural pre-induction phase in which bone marrow stem cells are forced to differentiate towards the neural stem or

progenitor cell phase. In the Jiang and Kabos methods the factors used in this pre-induction phase are growth factors used to culture neural stem cells. Therefore, in the case of NSC differentiation, this step could be omitted without any influence on the outcome of differentiation. In the neural induction phase of the Kohyama method, 5-azacytidine is used. This demethylating agent is believed to have enhancing effects on transdifferentiation of cells and has been used for transdifferentiation of bone marrow-derived cells to cardiomyocytes (Makino and others, 1999) and hepatocytes (Yamazaki and others, 2003). In our study, this factor was omitted since no transdifferentiation is needed in case of NSC differentiation. MAPC-like cells were differentiated using the protocols as originally described by the authors. The Jiang method has been optimized for neural differentiation of MAPC-like cells. The Kohyama method is designed for differentiating mesenchymal stem cells and the Kabos method uses unfractionated bone marrow as starting material.

Another neural differentiation method is a protocol described by Woodbury et al (Woodbury and others, 2000). Initially MAPC-like cells and NSC were compared in their potential for neural differentiation using this method but this protocol caused massive cell death of NSC (data not shown). This is in line with recent evidence showing that the adoption of neural morphology and the enhancement of neural gene expression in cells that are differentiated using the Woodbury protocol are likely to be stress artefacts (Neuhuber and others, 2004; Lu and others, 2004; Bertani and others, 2005). Therefore this differentiation protocol was omitted from this study.

In all three protocols, NSC differentiated towards cells with appropriate neural and glial morphology and positive for all neural or glial markers investigated with ICC and RT-PCR analysis. Semi-quantitative analysis showed that there was a clear augmentation in expression of three markers, tau, GFAP and MBP after differentiation. For the other two markers investigated, β 3-tub and NF200, expression levels did not change dramatically due to differentiation. These data demonstrate that each of the three protocols, as we performed them in the laboratory, can indeed induce neural differentiation.

Our MAPC-like cells express nestin at baseline, an intermediate filament protein predominantly expressed by neural stem cells. This baseline expression has also been demonstrated in standard rat bone marrow-derived stromal cells (Wislet-Gendebien and others, 2003). Although we would expect an augmentation of nestin expressing cells after the neural induction phase, we could not demonstrate this with our immunocytochemistry data. The proportion of cells, positive for nestin, was even less after predifferentiation than at baseline conditions. Further differentiating these “predifferentiated” cells resulted only in the Jiang method in cells with a bipolar neural-like morphology. In the other differentiation protocols, the cells formed extensions but adopted a more fibroblast-like morphology. MAPC-like cells were positive for two neural markers (β 3-tub and NF200) already at baseline but there was no clear upregulation of these genes after exposure to all differentiation protocols. In the case of tau, GFAP and MBP, cells did not express these markers before or after differentiation at levels detectable with RT-PCR or ICC.

In contrast to our study, the Minneapolis group did report the differentiation of bone marrow-derived MAPC cells towards cells positive for different neural, astrocyte and oligodendrocyte markers using the protocol we used in our study. This group succeeded in differentiating murine MAPC-like cells towards functional neurons using a complex co-culture differentiation protocol (Jiang and others, 2003). Our MAPC-like cells also had multipotent capacity since we were able to differentiate them towards different mesodermal cell fates. However, we did not succeed in differentiating rat MAPC-like cells towards neuroectodermal phenotypes on a persuasive basis, although several attempts were made to reproduce the neural differentiation results of the Minneapolis group. By comparing the outcome of the neural differentiation of the MAPC-like cells with that of a positive control, adult NSC, we demonstrate that the protocols used in this study can induce neural differentiation but only in cells that have this potential.

In summary, our results show that bone marrow-derived MAPC-like cells express “neural markers” nestin, β 3-tub and NF200 and can be induced to extend neurite-like extensions. However these factors cannot be seen as proof for a neuropotency since the differentiation protocols tested have no clear inducing or enhancing effects on the expression of neuro-glial genes in MAPC-like cells while, for β 3-tub and NF200, they do in the positive control NSC. Recent studies have demonstrated that the formation of neurite-like structures is not always the result of the development of axons or dendrites but can also be induced by exposing cells to several stressors (Neuhuber and others, 2004; Lu and others, 2004; Bertani and others, 2005). In all protocols used in this study to differentiate MAPC-like cells, the FCS used to culture MAPC-like cells is withdrawn from the medium. Several studies have demonstrated that removal of serum induces oxidative stress and DNA fragmentation. So as an alternative explanation, the formation of neurite-like extensions by MAPC-like cells in our study could be the result of stress induced by serum-withdrawal. There are also several indications why the expression of β 3-tub and NF200 is no proof for a true neural phenotype. First, the present study demonstrates that differentiated MAPC-like cells do not express other pan-neural or glial markers such as tau and GFAP or MBP; while these are expressed in differentiated NSC. Second, β 3-tub and NF200 but also other presumed neural-specific markers are not exclusively expressed in neural cells but also in hematopoietic progenitor cells (Goolsby and others, 2003) and undifferentiated bone marrow stromal cells (Tondreau and others, 2004; Bertani and others, 2005). Third, a recent study showed that it is possible to induce expression of neural specific antigens and neurotransmitters by exposing bone marrow cells to different mixes of growth factors. A closer evaluation, however, showed that the intracellular distribution of the antigens was different from that seen in adult neurons, implicating that these cells probably cannot function as true neurons (Jin and others, 2003).

The main conclusion of our study is that the expression of neural antigens by MAPC-like cells in this study probably is a phenomenon of aberrant expression rather than true transdifferentiation. We conclude this based on the fact that subjection of multipotent MAPC-like cells, to neural differentiation protocols, which were adopted from other transdifferentiation studies and proven to work for NSC, did not induce or upregulate expression of the analyzed neural and glial antigens. Our study does not rule out that MAPC or mesenchymal stem cells may have the potential to differentiate towards neural cells. The data, however, show very clearly

that validation of transdifferentiation of cells towards neural cell types, using only morphology and a limited set of neural antigen expression as outcome parameters, is not sufficient and that one has to be very rigorous in the interpretation of these neural transdifferentiation experiments. Furthermore, we show that the critical steps in neural differentiation of MAPC cells are not yet fully understood and undefined parameters may profoundly influence the results obtained in different laboratories.

REFERENCES

- Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A. 2003. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 425(6961):968-73.
- Bertani N, Malatesta P, Volpi G, Sonogo P, Perris R. 2005. Neurogenic potential of human mesenchymal stem cells revisited: analysis by immunostaining, time-lapse video and microarray. *J Cell Sci* 118(Pt 17):3925-36.
- Bossolasco P, Cova L, Calzarossa C, Rimoldi SG, Borsotti C, Delilieri GL, Silani V, Soligo D, Polli E. 2005. Neuroglial differentiation of human bone marrow stem cells in vitro. *Exp Neurol* 193(2):312-25.
- Brazelton TR, Rossi FM, Keshet GI, Blau HM. 2000. From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* 290(5497):1775-9.
- Castro RF, Jackson KA, Goodell MA, Robertson CS, Liu H, Shine HD. 2002. Failure of bone marrow cells to transdifferentiate into neural cells in vivo. *Science* 297(5585):1299.
- Cavaleri F, Schöler H. 2006. Molecular bases of pluripotency. In: Lanza R, Gearhart J, Hogan B, Melton D, Pedersen R, Thomson J, E.Donnall T, West M, editors. *Essentials of stem cell biology*. London: Elsevier Inc.; p 29-42.
- D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. 2004. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci* 117(Pt 14):2971-81.
- Deng W, Obrocka M, Fischer I, Prockop DJ. 2001. In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. *Biochem Biophys Res Commun* 282(1):148-52.
- Dewald GW, Broderick DJ, Tom WW, Hagstrom JE, Pierre RV. 1985. The efficacy of direct, 24-hour culture, and mitotic synchronization methods for cytogenetic analysis of bone marrow in neoplastic hematologic disorders. *Cancer Genet Cytogenet* 18(1):1-10.
- Dezawa M, Kanno H, Hoshino M, Cho H, Matsumoto N, Itokazu Y, Tajima N, Yamada H, Sawada H, Ishikawa H, Mimura T, Kitada M, Suzuki Y, Ide C. 2004. Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J Clin Invest* 113(12):1701-10.
- Eglitis MA, Mezey E. 1997. Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc Natl Acad Sci U S A* 94(8):4080-5.
- Gobbel GT, Choi SJ, Beier S, Niranjan A. 2003. Long-term cultivation of multipotential neural stem cells from adult rat subependyma. *Brain Res* 980(2):221-32.
- Goolsby J, Marty MC, Heletz D, Chiappelli J, Tashko G, Yarnell D, Fishman PS, Dhib-Jalbut S, Bever CT, Jr., Pessac B, Trisler D. 2003. Hematopoietic progenitors express neural genes. *Proc Natl Acad Sci U S A* 100(25):14926-31.
- Jiang Y, Henderson D, Blackstad M, Chen A, Miller RF, Verfaillie CM. 2003. Neuroectodermal differentiation from mouse multipotent adult progenitor cells. *Proc Natl Acad Sci U S A* 100 Suppl 1:11854-60.
- Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418(6893):41-9.
- Jin K, Mao XO, Batteur S, Sun Y, Greenberg DA. 2003. Induction of neuronal markers in bone marrow cells: differential effects of growth factors and patterns of intracellular expression. *Exp Neurol* 184(1):78-89.
- Kabos P, Ehtesham M, Kabosova A, Black KL, Yu JS. 2002. Generation of neural progenitor cells from whole adult bone marrow. *Exp Neurol* 178(2):288-93.
- Kim BJ, Seo JH, Bubien JK, Oh YS. 2002. Differentiation of adult bone marrow stem cells into neuroprogenitor cells in vitro. *Neuroreport* 13(9):1185-8.

- Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, Gojo S, Taga T, Okano H, Hata J, Umezawa A. 2001. Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. *Differentiation* 68(4-5):235-44.
- Levy YS, Merims D, Panet H, Barhum Y, Melamed E, Offen D. 2003. Induction of neuron-specific enolase promoter and neuronal markers in differentiated mouse bone marrow stromal cells. *J Mol Neurosci* 21(2):121-32.
- Liu S, Qu Y, Stewart TJ, Howard MJ, Chakraborty S, Holekamp TF, McDonald JW. 2000. Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. *Proc Natl Acad Sci U S A* 97(11):6126-31.
- Locatelli F, Corti S, Donadoni C, Guglieri M, Capra F, Strazzer S, Salani S, Del BR, Fortunato F, Bordoni A, Comi GP. 2003. Neuronal differentiation of murine bone marrow Thy-1- and Sca-1-positive cells. *J Hematother Stem Cell Res* 12(6):727-34.
- Lu P, Blesch A, Tuszynski MH. 2004. Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? *J Neurosci Res* 77(2):174-91.
- Magaki T, Kurisu K, Okazaki T. 2005. Generation of bone marrow-derived neural cells in serum-free monolayer culture. *Neurosci Lett* 384(3):282-7.
- Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. 1999. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest* 103(5):697-705.
- Massengale M, Wagers AJ, Vogel H, Weissman IL. 2005. Hematopoietic cells maintain hematopoietic fates upon entering the brain. *J Exp Med* 201(10):1579-89.
- Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. 2000. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* 290(5497):1779-82.
- Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B. 2003. Transplanted bone marrow generates new neurons in human brains. *Proc Natl Acad Sci U S A* 100(3):1364-9.
- Munoz-Elias G, Marcus AJ, Coyne TM, Woodbury D, Black IB. 2004. Adult bone marrow stromal cells in the embryonic brain: Engraftment, migration, differentiation, and long-term survival. *J Neurosci* 24(19):4585-95.
- Nakano K, Migita M, Mochizuki H, Shimada T. 2001. Differentiation of transplanted bone marrow cells in the adult mouse brain. *Transplantation* 71(12):1735-40.
- Neuhuber B, Gallo G, Howard L, Kostura L, Mackay A, Fischer I. 2004. Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. *J Neurosci Res* 77(2):192-204.
- Niwa H, Miyazaki J, Smith AG. 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 24(4):372-6.
- Padovan CS, Jahn K, Birnbaum T, Reich P, Sostak P, Strupp M, Straube A. 2003. Expression of neuronal markers in differentiated marrow stromal cells and CD133+ stem-like cells. *Cell Transplant* 12(8):839-48.
- Peinnequin A, Mouret C, Birot O, Alonso A, Mathieu J, Clarencon D, Agay D, Chancerelle Y, Multon E. 2004. Rat pro-inflammatory cytokine and cytokine related mRNA quantification by real-time polymerase chain reaction using SYBR green. *BMC Immunol* 5:3.
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science* 284(5411):143-7.
- Priller J, Persons DA, Klett FF, Kempermann G, Kreutzberg GW, Dirnagl U. 2001. Neogenesis of cerebellar Purkinje neurons from gene-marked bone marrow cells in vivo. *J Cell Biol* 155(5):733-8.
- Ratajczak MZ, Kucia M, Rea R, Majka M, Janowska-Wieczorek A, Ratajczak J. 2004. Stem cell plasticity revisited: CXCR4-positive cells expressing mRNA for early muscle, liver and neural cells 'hide out' in the bone marrow

- Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. 2001. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood* 98(9):2615-25.
- Reynolds BA, Weiss S. 1996. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* 175(1):1-13.
- Rismanchi N, Floyd CL, Berman RF, Lyeth BG. 2003. Cell death and long-term maintenance of neuron-like state after differentiation of rat bone marrow stromal cells: a comparison of protocols. *Brain Res* 991(1-2):46-55.
- Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. 2000. Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp Neurol* 164(2):247-56.
- Schwartz RE, Reyes M, Koodie L, Jiang Y, Blackstad M, Lund T, Lenvik T, Johnson S, Hu WS, Verfaillie CM. 2002. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J Clin Invest* 109(10):1291-302.
- Song HJ, Stevens CF, Gage FH. 2002. Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. *Nat Neurosci* 5(5):438-45.
- Toda H, Takahashi J, Mizoguchi A, Koyano K, Hashimoto N. 2000. Neurons generated from adult rat hippocampal stem cells form functional glutamatergic and GABAergic synapses in vitro. *Exp Neurol* 165(1):66-76.
- Tondreau T, Lagneaux L, Dejeneffe M, Massy M, Mortier C, Delforge A, Bron D. 2004. Bone marrow-derived mesenchymal stem cells already express specific neural proteins before any differentiation. *Differentiation* 72(7):319-26.
- Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. 2002. Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science* 297(5590):2256-9.
- Weimann JM, Johansson CB, Trejo A, Blau HM. 2003. Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant. *Nat Cell Biol* 5(11):959-66.
- Whittemore SR, Morassutti DJ, Walters WM, Liu RH, Magnuson DS. 1999. Mitogen and substrate differentially affect the lineage restriction of adult rat subventricular zone neural precursor cell populations. *Exp Cell Res* 252(1):75-95.
- Wislet-Gendebien S, Leprince P, Moonen G, Rogister B. 2003. Regulation of neural markers nestin and GFAP expression by cultivated bone marrow stromal cells. *J Cell Sci* 116(Pt 16):3295-302.
- Woodbury D, Reynolds K, Black IB. 2002. Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. *J Neurosci Res* 69(6):908-17.
- Woodbury D, Schwarz EJ, Prockop DJ, Black IB. 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 61(4):364-70.
- Yamazaki S, Miki K, Hasegawa K, Sata M, Takayama T, Makuuchi M. 2003. Sera from liver failure patients and a demethylating agent stimulate transdifferentiation of murine bone marrow cells into hepatocytes in coculture with nonparenchymal liver cells. *J Hepatol* 39(1):17-23.
- Zhao LR, Duan WM, Reyes M, Keene CD, Verfaillie CM, Low WC. 2002. Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Exp Neurol* 174(1):11-20.

MANUSCRIPT 4

*RAEDT R., VAN DYCKE A., VAN MELKEBEKE D., DE SMEDT T., CLAEYS P., WYCKHUYS T.,
VONCK K., WADMAN W. AND BOON P.*

**Seizures in the intrahippocampal kainic acid epilepsy model:
characterization using long term video-EEG monitoring in the rat.**

Acta Neurologica Scandinavica . 2007, submitted.

Seizures in the intrahippocampal kainic acid epilepsy model: characterization using long term video-EEG monitoring in the rat

Acta Neurologica Scandinavica (2007, submitted)

ROBRECHT RAEDT¹, ANNELIES VAN DYCKE¹, DEBORAH VAN MELKEBEKE¹,
TIM DE SMEDT¹, PIETER CLAEYS¹, TINE WYCKHUYS¹, KRISTL VONCK¹,
WYTSE WADMAN² AND PAUL BOON¹

¹Laboratory for Clinical and Experimental Neurophysiology, Department of Neurology, Ghent University Hospital, Ghent, Belgium

²Swammerdam Institute of Life Sciences, Department of Neurobiology, University of Amsterdam, The Netherlands

In this manuscript we describe the results of a long term video-EEG monitoring study of rats which had experienced a status epilepticus (SE) evoked by injection of kainic acid through a cannula in the hippocampus. The rats were monitored with video-EEG during two separate periods: the first 21 weeks after SE and week 40 to week 42 after SE. Characteristics of spontaneous seizures such as seizure duration, severity and frequency were determined. We investigated whether spontaneous seizures came in a circadian pattern and whether there was a progression of seizure severity.

SUMMARY

Intrahippocampal injection of kainic acid (KA) in rats evokes a status epilepticus and leads to spontaneous seizures. However to date, precise electroencephalographic and clinical characterization of spontaneous seizures in this epilepsy model using long-term video-EEG monitoring has not been performed.

Rats were implanted with bipolar hippocampal depth electrodes and a cannula for the injection of KA (0.4 µg/0.2 µl) in the right hippocampus. Video-EEG monitoring was used to determine habitual parameters of spontaneous seizures such as seizure frequency, severity, progression and day-night rhythms.

Spontaneous seizures were detected in all rats, with 13 out of 15 animals displaying seizures during the first eight weeks after status epilepticus. A considerable fraction (35%) of the spontaneous seizures did not secondary generalize. Seizure frequency was quite variable and the majority of the KA treated animals had less than one seizure per day. A circadian rhythm was observed in all rats that showed sufficient seizures per day.

This study shows that the characteristics of spontaneous seizures in the intrahippocampal KA model display many similarities to other SE models and to human TLE.

INTRODUCTION

Mesial temporal lobe epilepsy (TLE) is the most common form of symptomatic epilepsy and is characterized by complex partial seizures (with or without secondary generalization) originating from temporal lobe regions (Engel, 1996). TLE development covers three phases; 1) an initial precipitating insult of the brain such as febrile seizures, head trauma, SE, or stroke initiating a cascade of events; 2) a period of epileptogenesis during which several processes (either molecular or structural) occur; 3) chronic epilepsy characterized by the occurrence of spontaneous seizures.

Chronic TLE models need to resemble the epileptic state in which spontaneous and recurrent seizures occur (Mascott and others, 1994; Loscher, 1997). In rodents, spontaneous seizures that originate from the temporal lobe, can be provoked by brain infarction (Kelly and others, 2001; Kelly and others, 2006), traumatic brain damage (Kharatishvili and others, 2006), febrile seizures (Dube and others, 2006), kindling (Michael and others, 1998) and status epilepticus (SE). The SE model of chronic TLE has been extensively studied (Ben-Ari and others, 1980; French and others, 1982; Cavalheiro and others, 1983; Sater and Nadler, 1988; Lothman and others, 1989; Leite and others, 1990; Cavalheiro and others, 1991; Stafstrom and others, 1992; Tanaka and others, 1992; Mascott and others, 1994; Bertram and Cornett, 1994; Lemos and Cavalheiro, 1995; Leite and others, 1996; Hellier and others, 1998; Miettinen and others, 1998; Arida and others, 1999; Bragin and others, 1999; Nissinen and others, 2000; Gorter and others, 2001; Glien and others, 2001; Mazarati and others, 2002; Klitgaard and others, 2002; Furtado Mde and others, 2002; Brandt and others, 2003). In this model SE can be induced either by electrical or by chemical (kainic acid, pilocarpine) stimulation of various brain regions. Electrical stimulation has been applied to the perforant path (Gorter and others, 2001; Mazarati and others, 2002), the ventral hippocampus (Lothman and others, 1989; Bertram and Cornett, 1994), or the amygdala (Nissinen and others, 2000; Brandt and others, 2003). Pilocarpine (PILO), a chemoconvulsant, can be administered systemically or intracerebrally, both leading to spontaneous seizures in a high fraction of rats (Leite and others, 1990; Cavalheiro and others, 1991; Lemos and Cavalheiro, 1995; Arida and others, 1999; Glien and others, 2001; Klitgaard and others, 2002; Furtado Mde and others, 2002). Another frequently used chemoconvulsant, kainic acid (KA), is a structural analogue of glutamic acid and is isolated from the seaweed *Digenea simplex*. KA can be injected as a single bolus systemically or intracerebrally (Ben-Ari and others, 1980; French and others, 1982; Cavalheiro and others, 1983; Sater and Nadler, 1988; Stafstrom and others, 1992; Tanaka and others, 1992; Mascott and others, 1994; Leite and others, 1996; Hellier and others, 1998; Miettinen and others, 1998; Bragin and others, 1999). Direct injection of KA into specific brain regions, evokes SE at much lower doses and limits direct toxic effects to small brain areas. KA has been injected directly in the amygdala (Ben-Ari and others, 1980; Tanaka and others, 1992; Mascott and others, 1994), in the ventricle (Sater and Nadler, 1988), in the entorhinal cortex (Miettinen and others, 1998) and in the hippocampus (French and others, 1982; Cavalheiro and others, 1983; Leite and others, 1996; Bragin and others, 1999; Bragin and others, 2004; Bragin and others, 2005). These studies show that intracerebral injection of KA efficiently induces SE which on its turn induces epileptogenesis, that leads to spontaneous seizures.

For the intrahippocampal KA model electrophysiological features of epileptogenesis and spontaneous seizures resemble those of patients with refractory TLE (Bragin and others, 1999; Bragin and others, 2004). Long term video-EEG monitoring of the intrahippocampal KA model to determine habitual seizures parameters has not yet been performed. In this study, we injected KA in the hippocampus of unanaesthetized rats which were subsequently monitored for several months with video-EEG. We determined the duration of the SE, the fraction of rats displaying spontaneous seizures and seizure parameters such as seizure type, duration and frequency. We also assessed the progression in seizure occurrence and determined whether spontaneous seizures came in a circadian pattern.

MATERIALS AND METHODS

ANIMALS

Female Sprague-Dawley rats, weighting 200-220g (Harlan, Horst, The Netherlands), were kept under environmentally controlled conditions (12h normal light/dark cycles, 20-23°C and 50% relative humidity) with food and water ad libitum. The animals were treated according to guidelines approved by the European Ethics Committee (decree 86/609/EEC). The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University Hospital (ECP 05/38).

SURGERY

All rats (n=17) were anesthetized with an intraperitoneal injection of a ketamine (57 mg/kg) and xylazine (9 mg/kg) mixture. The rats were fixated in a stereotactic frame (Bilaney Consultants, Düsseldorf, Germany), the skull was exposed and nine holes were drilled to place the epidural and depth electrodes. Two custom-made epidural electrodes and one reference electrode (stainless steel screws, 1.57mm diameter, Bilaney, Germany) were placed on both sides of the skull over the frontal cortex and on the left side of the sutura lambdoidea respectively. Two polyimide coated stainless steel wires (70µm diameter, CFW, USA, distance between the two contact points: 0.5mm) were glued together to form bipolar electrodes. These depth electrodes were placed in both hippocampi (AP= - 5.6 mm, ML= ± 4.5 mm, DV=-5.5 mm relative to bregma). The electrode in the right hippocampus (fig 1a) was fixed to a stainless steel guide cannula (fig 1c; 0.81 mm o.d.; Plastics One, Sevenoaks, UK) using a shrinking tube (fig 1d), which was heated to fix the electrode to the cannula. The guide cannula was closed off using a dummy cannula (fig 1b; Plastics One). The distance between the tips of the electrode and the cannula was 3.5 mm. In this way the cannula ended just above the hippocampus preventing additional damage to the hippocampus (fig 1). In the remaining four burr holes, anchor screws were placed to fix the electrodes on the skull by acrylic cement. All electrodes were lead to a connector which was attached via a commutator to the EEG cables.

INTRAHIPPOCAMPAL KA INJECTION

One week after surgery the rats were manually restrained. The dummy cannula was removed and a 30 G needle was lowered in the guide cannula. A mechanical stop placed on the needle allowed positioning of the injection needle at the same depth of the lowest tip of the recording electrode in the hippocampus. In 15 rats, 0.4 µg/0.2 µl of KA (Ocean Produce International, Shelburne, Canada) was slowly injected (0.1 µl/min) into the right hippocampus using a 1µl Hamilton syringe attached to the needle (Sigma, Bornem, Belgium). In two rats saline (0.2 µl) was injected using the same protocol. After injection the needle was left in place for an additional minute after which it was slowly retracted and the cannula was closed off with a dummy cannula.

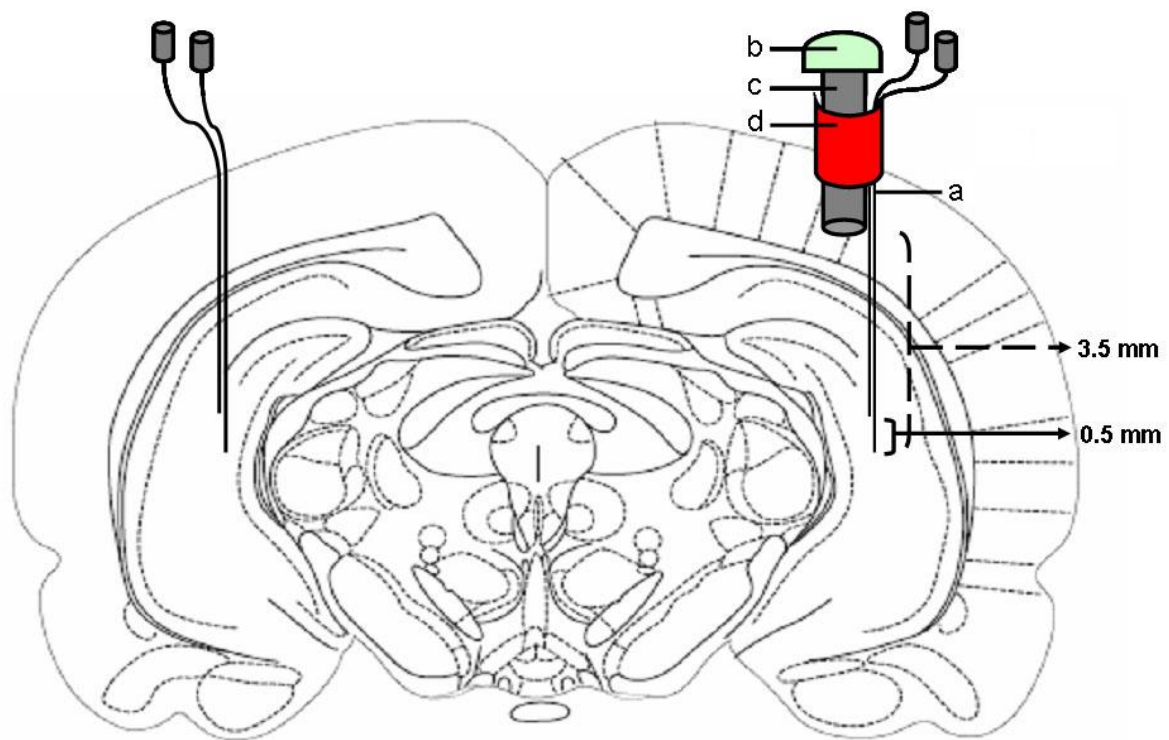


Fig 1: Schematic drawing of the stereotactic implantation of bipolar depth electrodes in the left hippocampus and a electrode-cannula complex in the right hippocampus: This image is a schematic overview of a coronal brain section 5.6 mm posterior to bregma (Paxinos and Watson, 1998), to illustrate the position of left and right hippocampal depth electrodes (ML: ± 4.5 mm, DV: -5.5 mm compared to bregma). The right bipolar hippocampal electrode (a) is combined to a guide cannula (c) using a shrinking tube (d) which is heated. The guide cannula is closed off using a dummy cannula (b). KA is injected through the guide cannula at the same depth of the most ventral electrode tip.

VIDEO-EEG-MONITORING

The video-EEG monitoring was performed under environmentally controlled conditions (12h normal light/dark cycles, 20-23°C and 50% relative humidity) in an isolated room. Lights were on from 8 AM until 8 PM. The unit existed of 8 custom-made cages. A 4-channel MOSFET input operational amplifier functioned as an impedance buffer and connected the electrodes on the head to the cable. The EEG signals were sampled at 200 Hz with 16 bit resolution (PCI-6033E, National Instruments, Texas, USA) and stored on a PC. Video was recorded using an infrared sensitive camera (CAMCOLBUL5, Velleman, Ghent, Belgium) coupled to a PCTV card (Miro Video Studio PCTV, Pinnacle Systems, California, USA) and was stored in a compressed format (MPEG-4) on a PC using a freeware program (VirtualDub version 1.6.14). This program marked date and time on the captured video so synchronizing video-images with EEG.

MONITORING OF STATUS EPILEPTICUS

In three consecutive sessions (8 KA, 7 KA and 2 controls) rats were monitored for seizure activity for 48 hours after injection of KA or saline. Epileptiform activity could not be detected in the EEG of the two rats injected with saline and these rats did not display clinical seizure activity. In the KA treated rats, we separately evaluated epileptic activity in the EEG and seizure semiology. We calculated the time between KA injection and

the appearance of epileptiform discharges on the hippocampal electrodes and on the epidural electrodes. The EEG duration of the SE was defined as the time between the occurrence of the first epileptiform discharges in the EEG and the time point after which no high frequency, high amplitude discharges were detected. Duration of the clinical, behavioural SE was defined on the basis of clear motor symptoms observable on the video images. In case of wild running and jumping of the animals we interrupted the SE with pentobarbital (Nembutal, 60 mg/kg, i.p.), as this behaviour often culminates in the death of the animal. Rats were left in the monitoring cages for 48 hours after KA injection for the continuous recording of video-EEG and were then transferred to their regular cages until the next monitoring session, one week after SE.

MONITORING OF SPONTANEOUS SEIZURES

The occurrence of spontaneous seizures was monitored for 21 weeks, during 72 hours per week, in all rats starting one week after SE. Forty weeks after SE, a second video-EEG monitoring period of 14 days was started in four surviving KA treated rats. The EEG of both monitoring periods was screened for spontaneous EEG seizures. An EEG seizure was defined as a period of consistent, repetitive changes in amplitude and frequency of electrical activity that persisted for more than 10 s (fig. 2). Video-images were evaluated for clinical behaviour in the periods where EEG seizures were detected and seizure semiology was scored using a modified scale of Racine (Racine, 1972). Seizures accompanied by immobility, facial clonus, wet dog shakes, head nodding, chewing (severity stage 1 and 2) were grouped as partial seizures. More severe seizures of stages 3, 4 or 5 were grouped as partial seizures with secondary generalization (SG) and defined as: stage 3: unilateral clonus of one forepaw, stage 4: bilateral clonus of forepaws and rearing, stage 5: bilateral clonus of forepaws, rearing and falling, tonic-clonic seizures. During this long term video-EEG monitoring we determined several seizure parameters: seizure type, seizure duration, seizure frequency, progression of seizure and variation in seizure occurrence related to circadian rhythm. Duration of the seizures was determined on the EEG (see fig 2 for a typical example). Seizure frequency was defined as the total number of detected seizures during the relevant period divided by the recording period in days, which started after the detection of the first seizure. The time course of seizure progression was assessed and we tested whether the occurrence of spontaneous seizures was related to a circadian rhythm.

DATA ANALYSIS AND STATISTICS

The recorded EEG was evaluated using custom made software programmed in LabView 7.0 (National Instruments). Statistical analysis was performed in SPSS version 12. Unless otherwise stated, all data are expressed as means and standard error of the mean. In several cases, assumptions for parametric statistics were not fulfilled and non-parametric Wilcoxon and Spearman rank tests were used. $P < 0.05$ was assumed to indicate a significant difference.

RESULTS

STATUS EPILEPTICUS

Fifteen unanaesthetized rats were restrained and injected with KA (0.4 µg/0.2 µl) through a cannula placed in the right hippocampus. All 15 injected rats displayed SE with repeated EEG epileptic discharges and recurrent motor seizures. The mean latency between KA injection and the appearance of the first epileptiform discharge on the hippocampal EEG was 2 ± 1 min. The first epileptic discharge was seen on the scalp electrodes after a mean latency of 21 ± 6 min. Clinically, behavioural arrest (sometimes with jumping behaviour) was seen after KA injection. Behavioural arrest gradually evolved towards discrete motor seizures. After a mean latency of 66 ± 15 minutes the first discrete motor seizure was observed. This behaviour further progressed towards rapidly recurring generalized tonic clonic convulsions. Later in time after KA injection, convulsions were spaced by longer intervals and disappeared. Two rats had to be injected with pentobarbital (Nembutal, 60 mg/kg, i.p.) during SE because they displayed extremely wild running and jumping. One animal was injected 130 min and the other one 210 min after the start of the SE. The duration of SE in these two rats was not included for further analysis. The mean duration of behavioural SE was 457 ± 38 minutes which was significantly shorter than the EEG duration of 603 ± 19 minutes ($p < 0.01$, Wilcoxon signed rank test). After the last EEG discharge epileptiform spikes were still evident for at least 24 hours.

SPONTANEOUS SEIZURES

SEIZURE TYPE

In 13 of the 15 KA treated rats spontaneous seizures were detected during the first 8 weeks that were monitored after SE. In the other two rats spontaneous seizures were found during the second monitoring period at 40 to 42 weeks after SE. The mean latency to the first detected seizure in these 13 rats was 28 ± 4 days. The first detected spontaneous seizure was a partial seizure with SG in 9 of the 15 rats. We detected a total of 1329 EEG seizures during both monitoring periods (fig 2). Clinically, 876 (66%) of the seizures were partial seizures with SG and 453 (34%) were partial seizures without SG. All rats, but one, displayed partial seizures without SG. By evaluating both seizure types in individual rats, we found that the number of detected partial seizures with SG per rat (median: 25, range: 1-280) was significantly higher ($p < 0.05$, Wilcoxon signed rank test) than the number of detected partial seizures without SG (median: 5, range: 0-213). We also found that the occurrence frequency of both seizure types in an individual rat was positively correlated (Spearman correlation coefficient, $\rho=0.76$, $p<0.01$). This implies that rats with a high number of partial seizures also have a lot of partial seizures with SG.

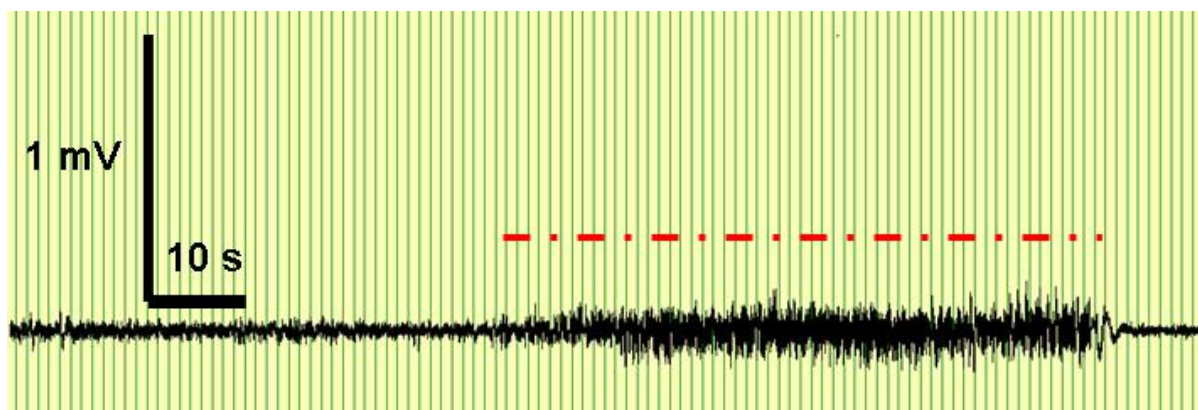


Fig 2: Example of EEG pattern of a spontaneous seizure in the intrahippocampal KA model: This EEG pattern is recorded using depth electrodes placed in the right (KA injected) hippocampus. The duration of the seizure was determined on the EEG by the time between the start and the end of the discharge (— —).

SEIZURE DURATION

The mean duration of spontaneous seizures in the KA treated rats was 64 ± 4 s. The duration of partial seizures was 46 ± 5 s which is significantly shorter than the duration of partial seizure with SG, which was 72 ± 7 s (Wilcoxon signed rank test, $p < 0.05$). Duration of spontaneous seizures during the first monitoring period did not change significantly over time and was not different from the duration of seizures in the second monitoring period.

SEIZURE FREQUENCY

As seizures are rare events, a large variation in the frequency of spontaneous seizures (expressed as seizures/day) was found among rats during the first monitoring period (range: 0-7.40 seizures per day, median: 0.56, Table 1). Four rats out of the 15 tested had more than one seizure per day and three out of 15 displayed more than three seizures per day (Table 1). No correlation was detected between SE duration (electrographic as well as clinical) and seizure frequency, but a negative correlation was detected between the latency to the first seizure detection and the seizure frequency (Spearman correlation coefficient, $\rho = -0.59$, $p < 0.05$).

During the second monitoring period, 40 weeks after KA injection, four of the fifteen rats were monitored again and this continuously for 14 days. Among these four rats there was again considerable variation in seizure frequency (range: 0.38-9.92 seizures per day, median: 0.95, Table 1).

Table 1: Mean daily seizure frequency for each individual rat during the first and second monitoring period.

	First monitoring period	Second monitoring period
Rat 1	7.40	<i>n.m.</i>
Rat 2	6.61	<i>n.m.</i>
Rat 3	3.22	<i>n.m.</i>
Rat 4	1.15	9.92
Rat 5	0.78	<i>n.m.</i>
Rat 6	0.75	<i>n.m.</i>
Rat 7	0.60	<i>n.m.</i>
Rat 8	0.56	<i>n.m.</i>
Rat 9	0.34	<i>n.m.</i>
Rat 10	0.20	<i>n.m.</i>
Rat 11	0.15	0.76
Rat 12	0.13	<i>n.m.</i>
Rat 13	0.08	<i>n.m.</i>
Rat 14	0.00	0.38
Rat 15	0.00	1.14

This table shows the mean daily frequency of spontaneous seizures measured during the first (left) and the second (right) monitoring period. In this table rats are vertically sorted depending on the daily frequency of total spontaneous seizures (upper rows: high frequency; lower rows: low frequency). Mean daily seizures frequency was calculated by dividing the total number of detected seizures by the number of days monitored starting from day of the first seizure detection. During the first monitoring period the median seizure frequency is 0.56 (range: 0.00-7.40 seizures/day). Three rats (1-3) displayed more than three seizures per day. One rat (4) had more than one but less than three seizures per day. Four rats (5-8) had at least one seizure every two days. Five rats (9-13) had less than one seizure in two days and in two rats (14-15) no seizures were detected during the first monitoring period. During the second monitoring period four of the fifteen rats were monitored again, but now continuously, for spontaneous seizures. For all four rats the daily seizure frequency was higher than in the first monitoring period. The median daily seizure frequency in this period was 0.95 seizures/day (range 0.38-9.92).

PROGRESSION OF EPILEPSY

Once seizures appear we generally observed a progression in seizure severity, frequency of occurrence and duration. As seizure frequency covered quite a large range of values we analysed the development over time of this variable only for the four rats that had sufficiently high seizure rates (a total of 715 seizures were used). There was a significant correlation between time and seizure frequency (Spearman correlation coefficient $\rho=0.47$, $p<0.05$, fig 3).

We also found a higher seizure frequency in the second monitoring period compared to the first in the four rats tested (table 1). Two rats, in which no spontaneous seizures were detected during the first monitoring session,

displayed 0.38 and 1.13 spontaneous seizures/day during the second monitoring period. In the two other KA treated rats an increase in spontaneous seizure frequency is seen compared to the last two weeks of the first monitoring session (9.92 compared to 1.15 seizures/day and 0.76 compared to 0.15 seizures/day).

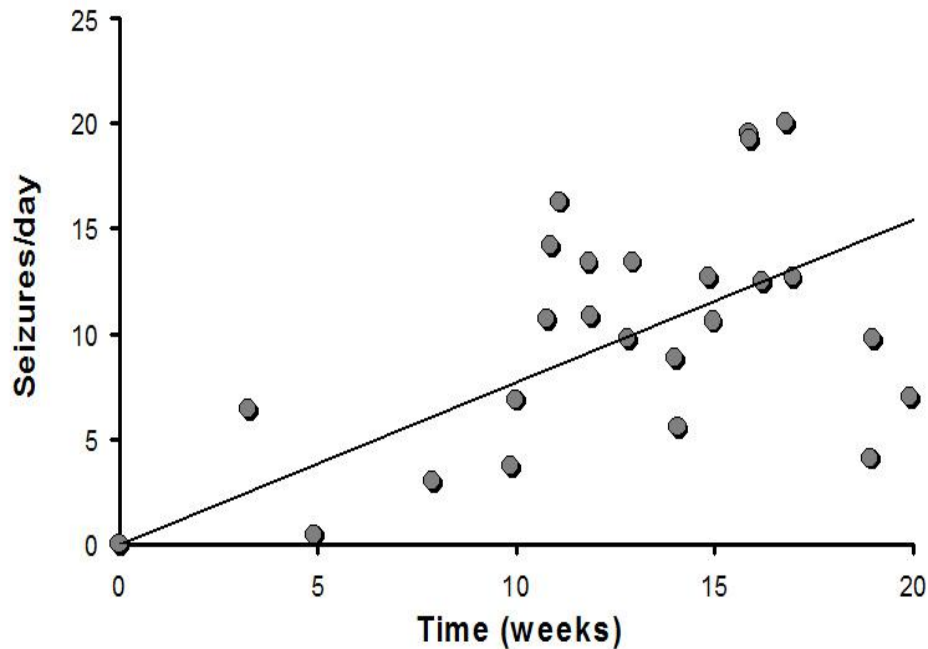


Fig 3: Seizure frequency in function of time: This graph shows seizure frequency (number of seizures per day) in function of time using four rats displaying in average more than one seizure per day. There is a significant positive correlation between seizure frequency and time ($\rho=0.47$, $p<0.05$). Individual data points on this graph represent daily seizure frequency determined for periods during which seizures frequencies remained relatively stable.

SEIZURE OCCURRENCE AND CIRCADIAN RHYTHM

The four rats, which displayed a relative high seizure rate, were also used to analyse a possible circadian component in seizure occurrence. The distribution of seizure occurrence as a function of time of day is illustrated in fig 4. A clear time lock with the day-light period could be found. The mean diurnal seizure frequency was 0.25 ± 0.02 seizures/hr, the mean nocturnal seizure frequency was 0.15 ± 0.01 seizures/hr. Both were significantly different from overall mean seizure frequency, being 0.20 ± 0.03 seizures/hr. Overall $62 \pm 1\%$ of the seizures occurred during the lights on period. In summary we found a clear circadian rhythm in the occurrence of spontaneous seizures with a significantly higher ($p<0.01$, Mann-Whitney U test) seizure frequency during the day (08:00-20:00) compared to seizure frequency during the night (20:00-08:00) with the highest seizure frequency between 14:00 and 16:00.

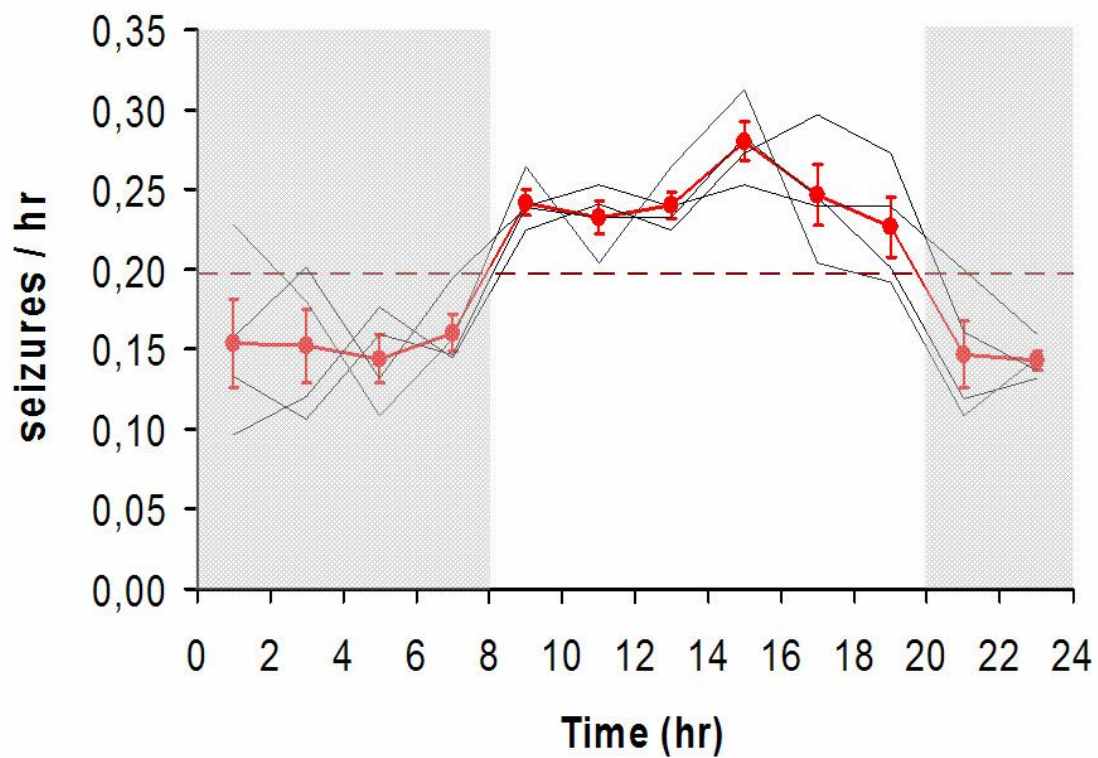


Fig 4: Circadian rhythm of spontaneous seizures: Based on the four rats, displaying in average more than one seizure per day, the mean seizure frequency (seizures/hour) was determined for each two hour segment of the day (●). From this graph it is evident that there is a circadian rhythm in the occurrence of seizures with significantly higher seizure frequencies during the day (08:00-20:00) compared to frequencies during the night (20:00-08:00; ▨). Both mean seizure frequency during the day (0.25 ± 0.02 seizures/hr) and during the night (0.15 ± 0.01 seizures/hr) were significantly different from the mean overall seizure frequency per two hours (0.20 ± 0.04 seizures/hr; ■■■). Seizure frequencies of the four individual rats were also displayed in this graph (—).

DISCUSSION

This study confirms that injection of KA directly in the hippocampus of unanaesthetized rats induces a SE in all rats (Bragin and others, 2004). This is not the case in other SE models, using excitotoxins or continuous electrical stimulation to evoke SE. In the intraperitoneal KA model, 93% (Stafstrom and others, 1992), in the intraperitoneal PILO model, 83% (Mello and others, 1993), in the intrahippocampal PILO model, 71%, (Furtado Mde and others, 2002), in the continuous hippocampal and perforant path stimulation model, 70% (Bertram and Cornett, 1994; Gorter and others, 2001) and in the amygdala stimulation model 87% (Nissinen and others, 2000) of the animals developed SE.

We found that the EEG duration of the SE after intrahippocampal KA injection was about 10 hours. The clinical duration of the SE, assessed by the occurrence of motor seizures, was significantly shorter being only about 7 to 8 hours. After continuous hippocampal stimulation the length of SE is between 6 and 12 hours as assessed by behavioural and electrographic monitoring (Mathern and others, 1997), in the continuous perforant path stimulation SE model total time spent in EEG seizures was 9 h (Mazarati and others, 2002) and in the continuous amygdala stimulation model about 12 hours (Nissinen and others, 2000). In a lot of studies on the SE model status is interrupted after a certain amount of time with diazepam or phenobarbital (Lemos and Cavalheiro, 1995; Gorter and others, 2001; Glien and others, 2001; Klitgaard and others, 2002; Furtado Mde and others, 2002). Overall, the duration of SE, when not interrupted, seems to be independent of the induction method.

We showed that SE, induced by intrahippocampal KA injection, leads to spontaneous seizures in all rats. In the majority of the animals (87%), seizures were detected during the first eight monitoring weeks. Analogue high fractions of animals with spontaneous seizures were reported after SE evoked by intraperitoneal injection of multiple low doses KA (97%) and PILO (100%) (Hellier and others, 1998; Glien and others, 2001) and after SE evoked by continuous electrical stimulation in the amygdala (87%), the hippocampus (100%) or the perforant path (100%) (Bertram and Cornett, 1994; Nissinen and others, 2000; Gorter and others, 2001). In humans the risk for spontaneous seizures after SE is lower being 37% within one year and 56 % within 3 years (Hauser and others, 1990). This lower risk is most likely a reflection of the immediate attempts to interrupt SE in patients and the chronic treatment of post-SE patients with AED.

We demonstrated that about 30% of the spontaneous seizures were partial without SG. After intrahippocampal PILO administration 16% of the partial seizures did not secondary generalize (Furtado Mde and others, 2002). In the continuous amygdala stimulation model, the fraction of partial seizures without SG increased with time: from 21% during the first 10 weeks after SE to 77% in the following weeks (Nissinen and others, 2000). In the continuous perforant path stimulation model a similar increase in the fraction of partial seizures, that did not generalize, was reported about 2 months after SE (Gorter and others, 2001). In our study we could not detect an increase of the proportion of partial seizures without SG with time. In human refractory TLE, seizures are mainly partial without SG since many AED control SG much better than partial seizures (Wieser, 2004).

Therefore TLE models that allow screening of partial seizures without SG are very useful for the development of new treatments of refractory TLE.

We found that mean seizure duration was about 64 s with secondary generalized seizures taking longer than non secondary generalized seizures. In the intraperitoneal KA model seizures last for about 30 s (Leite and others, 1990). In the intraperitoneal PILO model seizure duration varied between 30s and 60 s (Leite and others, 1990; Glien and others, 2001). In the continuous hippocampal stimulation model seizures are recorded with a duration between 64 and 106 s (Bertram and Cornett, 1994). In the perforant path stimulation model seizures last up to 120 s (Mazarati and others, 1998; Gorter and others, 2001). In the amygdala stimulation model seizures last in average for 49 s (Nissinen and others, 2000). In TLE patients, it has been reported that partial seizures with SG typically last for about 1 minute (Theodore and others, 1994). Overall, duration of seizures is rather similar among the different experimental models and humans with TLE.

The daily frequency of spontaneous seizures varied considerably among the KA treated animals and varied from very few to 7 seizures per day. We showed that 20% of the animals displayed frequent seizures with more than three seizures per day, while 73% of the animals had a low seizure frequency with less than one seizure per day. Other studies have also reported that post-SE rats can be divided into frequently and rare seizing rats. In the continuous amygdala stimulation model, 31% of the animals display frequent seizures (Nissinen and others, 2000). In the perforant path stimulation model, frequent seizures (four or more per day) are detected in 67% to 76% of the rats (Mazarati and others, 1998; Gorter and others, 2001).

As reported by Mazarati and coworkers we could not detect a correlation between the duration of the SE and the daily frequency of spontaneous seizures (Mazarati and others, 2002). We did find that a short latency to detection of the first spontaneous seizure was an indicator for a high frequency of spontaneous seizures. This was also reported in the continuous amygdala stimulation model (Nissinen and others, 2000).

The daily frequency of convulsive seizures increases systematically during the first twenty weeks after SE, be it with a large variation within and among animals. In the intraperitoneal KA model a progression in the number of convulsive seizures is seen from 2 to 6 months after kainite treatment (Hellier and others, 1998). In the intraperitoneal PILO model a significant increase in the number of seizures was seen during the first two months after SE (Arida and others, 1999). In the continuous hippocampal stimulation model an increase in seizure frequency was reported during the three months after the first seizure (Bertram and Cornett, 1994). In the continuous perforant path stimulation model progression of seizure frequency was seen in the first two months after SE but only in a subgroup of rats. The authors demonstrate that this progression is associated with loss of hilar interneurons combined with mossy fiber sprouting (Gorter and others, 2001). The progression of seizure frequency in our and other models parallels the situation in most human patients suffering from TLE in whom a worsening of the epileptic state over time has also been described (Engel, 1996; Wieser, 2004).

In our study, we found that seizure frequency has a link to the circadian rhythm with the seizures occurring more frequently during the day (lights on) compared to the night (lights off). The highest seizure rate, although

not significant, was found in the period between 14:00 and 16:00. Overall $62 \pm 1\%$ of the seizures occurred during the day. The same tendency was found in the continuous hippocampal stimulation model where about 65% of the seizures occurred during the day with a peak occurrence at 15:00 (Quigg and others, 1998). Also in the amygdala stimulation model and the intraperitoneal KA model it was found that a higher fraction (respectively 57% and 64%) of the seizures occurred during the day (Hellier and Dudek, 1999; Nissinen and others, 2000). Hellier and coworkers normalized seizure occurrence for the activity-state of the animal and they found that more seizures come in periods of inactivity (82%) compared to periods of activity (18%) (Hellier and Dudek, 1999). Remarkably, spontaneous seizures in TLE patients preferentially occur during the day with a peak between 13:30 and 16:30, a period of the day during which humans are most active (Quigg and others, 1998).

CONCLUSION

To our knowledge this is the first report in which long term video-EEG monitoring is performed for the characterization of spontaneous seizures in the intrahippocampal KA model. We found that intrahippocampal KA induces SE and spontaneous seizures in each animal. By using video and EEG monitoring we showed that a high fraction of the seizures were partial without SG. We found that seizure frequency was quite variable with a limited fraction of rats displaying a high seizure frequency. In these frequently seizing rats a progression of the epileptic state and a circadian rhythm in seizure occurrence was found. All seizure characteristics investigated in this study corresponded with commonly found properties of seizures in experimental models and human patients with TLE. Therefore we suggest that the intrahippocampal kainic acid model is a valuable alternative model for spontaneous temporal lobe seizures.

REFERENCES

- Arida RM, Scorza FA, Peres CDA, Cavalheiro EA. 1999. The course of untreated seizures in the pilocarpine model of epilepsy. *Epilepsy Research* 34(2-3):99-107.
- Ben-Ari Y, Tremblay E, Ottersen OP. 1980. Injections of kainic acid into the amygdaloid complex of the rat: An electrographic, clinical and histological study in relation to the pathology of epilepsy. *Neuroscience* 5(3):515-28.
- Bertram EH, Cornett JF. 1994. The evolution of a rat model of chronic spontaneous limbic seizures. *Brain Research* 661(1-2):157-62.
- Bragin A, Azizyan A, Almajano J, Wilson CL, Engel J, Jr. 2005. Analysis of chronic seizure onsets after intrahippocampal kainic acid injection in freely moving rats. *Epilepsia* 46(10):1592-8.
- Bragin A, Engel J, Jr., Wilson CL, Vizenin E, Mathern GW. 1999. Electrophysiologic analysis of a chronic seizure model after unilateral hippocampal KA injection. *Epilepsia* 40(9):1210-21.
- Bragin A, Wilson CL, Almajano J, Mody I, Engel J, Jr. 2004. High-frequency Oscillations after Status Epilepticus: Epileptogenesis and Seizure Genesis. *Epilepsia* 45(9):1017-23.
- Brandt C, Glien M, Potschka H, Volk H, Loscher W. 2003. Epileptogenesis and neuropathology after different types of status epilepticus induced by prolonged electrical stimulation of the basolateral amygdala in rats. *Epilepsy Res* 55(1-2):83-103.
- Cavalheiro EA, de Feo MR, Mecarelli O, Ricci GF. 1983. Intracortical and intrahippocampal injections of kainic acid in developing rats: an electrographic study. *Electroencephalogr Clin Neurophysiol* 56(5):480-6.
- Cavalheiro EA, Leite JP, Bortolotto ZA, Turski WA, Ikonomidou C, Turski L. 1991. Long-term effects of pilocarpine in rats: structural damage of the brain triggers kindling and spontaneous recurrent seizures. *Epilepsia* 32(6):778-82.
- Dube C, Richichi C, Bender RA, Chung G, Litt B, Baram TZ. 2006. Temporal lobe epilepsy after experimental prolonged febrile seizures: prospective analysis. *Brain* 129(4):911-22.
- Engel J. 1996. Surgery for Seizures. *The New England Journal of Medicine* 334(10):647-53.
- French ED, Aldinio C, Schwarcz R. 1982. Intrahippocampal kainic acid, seizures and local neuronal degeneration: relationships assessed in unanesthetized rats. *Neuroscience* 7(10):2525-36.
- Furtado Mde A, Braga GK, Oliveira JA, Del Vecchio F, Garcia-Cairasco N. 2002. Behavioral, morphologic, and electroencephalographic evaluation of seizures induced by intrahippocampal microinjection of pilocarpine. *Epilepsia* 43(Suppl 5):37-9.
- Glien M, Brandt C, Potschka H, Voigt H, Ebert U, Loscher W. 2001. Repeated low-dose treatment of rats with pilocarpine: low mortality but high proportion of rats developing epilepsy. *Epilepsy Research* 46(2):111-9.
- Gorter JA, van Vliet EA, Aronica E, Lopes da Silva FH. 2001. Progression of spontaneous seizures after status epilepticus is associated with mossy fibre sprouting and extensive bilateral loss of hilar parvalbumin and somatostatin-immunoreactive neurons. *European Journal of Neuroscience* 13(4):657-69.
- Hauser WA, Rich SS, Annegers JF, Anderson VE. 1990. Seizure recurrence after a 1st unprovoked seizure: an extended follow-up. *Neurology* 40(8):1163-70.
- Hellier JL, Dudek FE. 1999. Spontaneous motor seizures of rats with kainate-induced epilepsy: effect of time of day and activity state. *Epilepsy Res* 35(1):47-57.
- Hellier JL, Patrylo PR, Buckmaster PS, Dudek FE. 1998. Recurrent spontaneous motor seizures after repeated low-dose systemic treatment with kainate: assessment of a rat model of temporal lobe epilepsy. *Epilepsy Res* 31(1):73-84.
- Kelly KM, Jukkola PI, Kharlamov EA, Downey KL, McBride JW, Strong R, Aronowski J. 2006. Long-term video-EEG recordings following transient unilateral middle cerebral and common carotid artery occlusion in Long-Evans rats. *Experimental Neurology* 201(2):495-506.

- Kelly KM, Kharlamov A, Hentosz TM, Kharlamova EA, Williamson JM, Bertram EH, III, Kapur J, Armstrong DM. 2001. Photothrombotic brain infarction results in seizure activity in aging Fischer 344 and Sprague Dawley rats. *Epilepsy Res* 47(3):189-203.
- Kharatishvili I, Nissinen JP, McIntosh TK, Pitkanen A. 2006. A model of posttraumatic epilepsy induced by lateral fluid-percussion brain injury in rats. *Neuroscience* 140(2):685-97.
- Klitgaard H, Matagne A, Vanneste-Goemaere J, Margineanu DG. 2002. Pilocarpine-induced epileptogenesis in the rat:: Impact of initial duration of status epilepticus on electrophysiological and neuropathological alterations. *Epilepsy Research* 51(1-2):93-107.
- Leite JP, Babb TL, Pretorius JK, Kuhlman PA, Yeoman KM, Mathern GW. 1996. Neuron loss, mossy fiber sprouting, and interictal spikes after intrahippocampal kainate in developing rats. *Epilepsy Res* 26(1):219-31.
- Leite JP, Bortolotto ZA, Cavalheiro EA. 1990. Spontaneous recurrent seizures in rats: an experimental model of partial epilepsy. *Neurosci Biobehav Rev* 14(4):511-7.
- Lemos T, Cavalheiro EA. 1995. Suppression of pilocarpine-induced status epilepticus and the late development of epilepsy in rats. *Exp Brain Res* 102(3):423-8.
- Loscher W. 1997. Animal models of intractable epilepsy. *Prog Neurobiol* 53(2):239-58.
- Lothman EW, Bertram EH, Bekenstein JW, Perlin JB. 1989. Self-sustaining limbic status epilepticus induced by 'continuous' hippocampal stimulation: electrographic and behavioral characteristics. *Epilepsy Res* 3(2):107-19.
- Mascott CR, Gotman J, Beaudet A. 1994. Automated EEG monitoring in defining a chronic epilepsy model. *Epilepsia* 35(4):895-902.
- Mathern GW, Bertram III EH, Babb TL, Pretorius JK, Kuhlman PA, Spradlin S, Mendoza D. 1997. In contrast to kindled seizures, the frequency of spontaneous epilepsy in the limbic status model correlates with greater aberrant fascia dentata excitatory and inhibitory axon sprouting, and increased staining for N-methyl--aspartate, AMPA and GABAA receptors. *Neuroscience* 77(4):1003-19.
- Mazarati A, Bragin A, Baldwin R, Shin D, Wilson C, Sankar R, Naylor D, Engel J, Wasterlain CG. 2002. Epileptogenesis after self-sustaining status epilepticus. *Epilepsia* 43(Suppl 5):74-80.
- Mazarati AM, Wasterlain CG, Sankar R, Shin D. 1998. Self-sustaining status epilepticus after brief electrical stimulation of the perforant path. *Brain Research* 801(1-2):251-3.
- Mello LE, Cavalheiro EA, Tan AM, Kupfer WR, Pretorius JK, Babb TL, Finch DM. 1993. Circuit mechanisms of seizures in the pilocarpine model of chronic epilepsy: cell loss and mossy fiber sprouting. *Epilepsia* 34(6):985-95.
- Michael M, Holsinger D, Ikeda-Douglas C, Cammisuli S, Ferbinteanu J, DeSouza C, DeSouza S, Fecteau J, Racine RJ, Milgram NW. 1998. Development of spontaneous seizures over extended electrical kindling: I. Electrographic, behavioral, and transfer kindling correlates. *Brain Research* 793(1-2):197-211.
- Miettinen R, Kotti T, Tuunanen J, Toppinen A, Riekkinen S, Halonen T. 1998. Hippocampal damage after injection of kainic acid into the rat entorhinal cortex. *Brain Research* 813(1):9-17.
- Nissinen J, Halonen T, Koivisto E, Pitkanen A. 2000. A new model of chronic temporal lobe epilepsy induced by electrical stimulation of the amygdala in rat. *Epilepsy Res* 38(2-3):177-205.
- Paxinos G, Watson C. 1998. *The Rat Brain in stereotaxic coordinates*. Fourth Edition ed. San Diego: Academic Press.
- Quigg M, Straume M, Menaker M, Bertram EH. 1998. Temporal distribution of partial seizures: Comparison of an animal model with human partial epilepsy. *Annals of Neurology* 43(6):748-55.
- Racine RJ. 1972. Modification of seizure activity by electrical stimulation: II. Motor seizure. *Electroencephalography and Clinical Neurophysiology* 32(3):281-94.
- Sater RA, Nadler JV. 1988. On the relation between seizures and brain lesions after intracerebroventricular kainic acid. *Neuroscience Letters* 84(1):73-8.
- Stafstrom CE, Thompson JL, Holmes GL. 1992. Kainic acid seizures in the developing brain: status epilepticus and spontaneous recurrent seizures. *Brain Res Dev Brain Res* 65(2):227-36.

Tanaka T, Tanaka S, Fujita T, Takano K, Fukuda H, Sako K, Yonemasu Y. 1992. Experimental complex partial seizures induced by a microinjection of kainic acid into limbic structures. *Progress in Neurobiology* 38(3):317-34.

Theodore WH, Porter RJ, Albert P, Kelley K, Bromfield E, Devinsky O, Sato S. 1994. The secondarily generalized tonic-clonic seizure: a videotape analysis. *Neurology* 44(8):1403-7.

Wieser HG. 2004. Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis. *Epilepsia* 45(6):695-714.

MANUSCRIPT 5

RAEDT R., VAN DYCKE A., WAEYTENS A., VONCK K., WADMAN W., BOON P.

**Transplantation of adult subventricular zone-derived neural stem cells in the kainic
acid lesioned hippocampus of the rat.**

Experimental Neurology. 2007,**submitted.**

Transplantation of adult subventricular zone derived neural stem cells in the kainic acid lesioned hippocampus of the rat

Experimental Neurology (submitted)

ROBRECHT RAEDT^{1,*}, ANNELIES VAN DYCKE^{1,*}, ANOUK WAEYTENS²,
KRISTL VONCK¹, WYTSE WADMAN³, PAUL BOON¹

¹Laboratory for Clinical and Experimental Neurophysiology, Department of Neurology, Ghent University Hospital, Belgium

²Department of Pathology, Ghent University Hospital, Belgium

³Swammerdam Institute of Life Sciences, Department of Neurobiology, University of Amsterdam, The Netherlands

** These authors contributed equally to this work*

In this study we explore the potential of adult neural stem cells (NSC) after transplantation in an experimental model for temporal lobe epilepsy. We describe the isolation of NSC from the subventricular zone of adult GFP-reporter mice, their expansion as neurospheres in culture and their transplantation into the sclerotic hippocampus of the intraventricular kainic acid rat model. Three and six weeks after transplantation we evaluated the survival, dispersion and differentiation of the transplanted NSC. To assess the effect of the kainic acid lesion on the outcome of NSC transplantation we also transplanted NSC into the intact hippocampus of control rats. We evaluated the effect of a longer delay between lesion and grafting by transplanting the NSC with a postlesion delay of 3 days and 3 weeks.

SUMMARY

Transplantation of cell lines secreting seizure modifying substances has lead to the suppression of seizures in models for temporal lobe epilepsy (TLE). However, the inability of these cell lines to survive long-term after transplantation makes the search for alternative cell sources mandatory. In this study transplantation of adult-derived neural stem cells (NSC) was evaluated in the intrahippocampal kainic acid (KA) model for TLE.

NSC, derived from the subventricular zone (SVZ) of transgenic green fluorescent protein (GFP) reporter mice and expanded as neurospheres, were transplanted in the intact and sclerotic hippocampus of respectively control (Norm group) and intrahippocampal KA injected rats and thisthree days (KA3d group) and three weeks (KA3w group) after KA injection. Survival, dispersion and differentiation of NSC were compared between the different groups, three and six weeks after transplantation.

In the Norm and both KA groups a small but comparable fraction of GFP expressing NSC survived for at least six weeks after transplantation with a higher and more robust survival rate in the KA3d compared to the KA3w group. In both KA groups cell dispersion was significantly lower than in the Norm group. In all three groups cells mainly differentiated towards astrocytes but the astrocytic differentiation was significantly stimulated in both KA groups.

Our study indicates that adult-derived NSC are able to survive long-term upon transplantation in the hippocampus where they seem to contribute to the astrogliotic response on the KA lesion. These results indicate that NSC could be further investigated for local delivery of seizure modifying substances in the sclerotic hippocampus.

INTRODUCTION

Epilepsy is a chronic disorder of the central nervous system, affecting 1% of the human population. It is characterized by the abnormal and synchronized firing of a large number of neurons, resulting in various clinical symptoms. Although treatment with antiepileptic drugs (AED) is effective in the majority of patients, ~30% remains medically refractory (Kwan and Brodie, 2000). Temporal lobe epilepsy (TLE) is the most common form of epilepsy in adults, with many patients being refractory (60%-75%) (Spencer, 2002). Epilepsy surgery is an invasive but often curative treatment option that aims at removing the ictal onset zone believed to be responsible for seizure occurrence (Wiebe and others, 2001). However, in a substantial number of patients surgery is not an option due to a potential risk of compromising normal brain function (Clusmann and others, 2002). For these patients the ongoing search for alternative treatments such as newly developed AED, dietary treatments, transcranial magnetic stimulation and neurostimulation may prove successful (Freeman and others, 1998; Handforth and others, 1998; Vonck and others, 2002; Velasco and others, 2005). Next to these recently developed therapies, cell transplantation has also been suggested as a potentially valuable alternative to temporal lobe resection (Bjorklund and Lindvall, 2000; Nilsen and Cock, 2004).

In different animal models for TLE neuronal excitability was decreased by transplanting fetal cells releasing antiepileptic substances such as GABA (Loscher and others, 1998; Ross and others, 2002), noradrenaline (Bortolotto and others, 1990; Bengzon and others, 1990; Lindvall and others, 1990; Kokaia and others, 1994) and acetylcholine (Ferencz and others, 1998; Ferencz and others, 2001). Since this approach involves the need of a high number of fetuses it is associated with a lot of ethical and practical issues which may always restrict the clinical application of such treatments.

Based on the promising results of fetal tissue transplantation for the local delivery of epilepsy modifying substances, other cell types have been engineered to secrete inhibitory substances. In a series of experiments, Boison and colleagues induced inactivation of adenosine metabolizing enzymes in different self-renewing cell types to generate adenosine overexpressing cell lines. Adenosine overexpressing fibroblasts, myoblasts and embryonic stem cell-derived glia, encapsulated in semi-permeable polymers were grafted in the lateral ventricles of fully kindled rats resulting in a complete suppression of seizures (Huber and others, 2001; Fedele and others, 2004; Guttinger and others, 2005a; Guttinger and others, 2005b). Thompson and coworkers engineered a conditionally immortalized mouse neuronal cell line to deliver GABA by driving GAD₆₅ expression using a doxycycline controlled promoter. Grafting of these cells into the anterior substantia nigra (SNr) (Thompson and others, 2000; Thompson and Suchomelova, 2004), the pyriform cortex (Gernert and others, 2002) and the dentate gyrus of the hippocampus (Thompson, 2005) of rats had clear antiepileptic effects in both the kindling and status epilepticus (SE) model.

An important limitation of the studies described above was that the cell lines were unable to survive long-term after transplantation even when they were not engineered to overexpress the antiepileptic substance. As a

consequence the antiepileptic effect was only temporary (Huber and others, 2001; Thompson, 2005; Guttinger and others, 2005a; Guttinger and others, 2005b).

Neural stem cells (NSC) are a heterogeneous population of self-renewing and multipotent cells found in embryonic brain but also in different regions of the adult brain including the hippocampus, the subventricular zone (SVZ), the striatum, the substantia nigra, the cortex, the spinal cord, the septum and the optic nerve (Lois and Alvarez-Buylla, 1993; Palmer and others, 1995; Gage and others, 1995; Weiss and others, 1996; Shihabuddin and others, 1997; Palmer and others, 1999; Gage, 2000; Lie and others, 2002). NSC have been successfully isolated from the rodent and the human brain (Palmer and others, 1995; Weiss and others, 1996; Gritti and others, 1999; Nunes and others, 2003; Westerlund and others, 2005). A widely used method for the expansion of NSC is to grow them as free floating aggregates, termed neurospheres (Reynolds and Weiss, 1996; Weiss and others, 1996). *In vitro* these neurosphere cultures are able to generate both neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1996; Weiss and others, 1996; Svendsen and others, 1999; Vescovi and others, 1999; Gobbel and others, 2003). Neurosphere-derived cells are able to survive for prolonged time periods after transplantation in the brain where they are able to generate both neurons and glia especially in neurogenic regions such as the SVZ and the hippocampus (Soares and Sotelo, 2004; Muraoka and others, 2006). There are also indications that transplanted NSC are able to survive in brains affected by a lesion (Herrera and others, 1999; Dziewczapolski and others, 2003; Kelly and others, 2004; Richardson and others, 2005a).

Intrahippocampal injection of kainic acid (KA) in rats causes SE and spontaneous seizures after a latent period (Raedt and others, 2007). Histopathological changes caused by KA injection and the subsequent status epilepticus resembles hippocampal sclerosis, which is characterized by selective neuronal cell death and extensive gliosis (Engel, Jr., 2001).

In this study, we investigated whether SVZ-derived NSC were able to survive long-term after transplantation in the sclerotic hippocampus of the intrahippocampal KA rat model for TLE. To determine the effect of the sclerotic lesion on the survival, dispersion and differentiation of the NSC, we compared the results with outcome of transplantation in a non-sclerotic hippocampus of control rats. To assess whether outcome parameters changed over time rats were killed three weeks and six weeks after transplantation. We also evaluated the effect of a longer delay between the lesion and the transplantation by grafting the cells both 3 days and 3 weeks after KA injection.

MATERIALS AND METHODS

All animal procedures and experiments were approved by the local Ethical Committee of the University of Ghent (ECP05-38). The animals were kept under environmentally controlled conditions (12h normal light/dark cycles, 20-23°C and 50% relative humidity) with food and water ad libitum.

ISOLATION OF NEURAL STEM CELLS

NSC were isolated from transgenic green fluorescent protein (GFP) mice according to a previously reported protocol (Raedt et al., 2006). In these mice ubiquitous expression of GFP is seen in all tissues because the GFP coding region is placed under the transcriptional control of the chicken β -actin promoter and human cytomegalovirus enhancer (Manfra and others, 2001). In brief, two male mice (20g) were sacrificed with pentobarbital overdose (Nembutal, Sanofi Santé B.V., France; 180 mg/kg). Their brains were removed and placed in artificial cerebrospinal fluid (124 mM NaCl, 5 mM KCl, 3.2 mM $MgCl_2$, 105 mM $NaHCO_3$, 10 mM glucose and 2 mM $CaCl_2$). The SVZ was microdissected bilaterally and dissociated by placing it for 45 minutes into a digestion medium containing 0.5mM Na_2EDTA (Sigma, Bornem, Belgium), 1.0 mM cysteine (Sigma), 0.9 mg/ml papain (Worthington, Lakewood, NJ) and 1 mg/ml DNase (Sigma), dissolved in Earle's balanced salt solution. Digestion was stopped by adding ovomucoid (0.5 mg/ml, Sigma). After mechanically dissociating the tissue, cells were plated in a 96-well flat-bottomed plate at a density of 10,000 cells per well in a chemically defined medium. The NSC growth medium consisted of NS-A medium (Euroclone, Milan, Italy) with an additional 2 mM L-glutamine (Cambrex, Verviers, Belgium), 3 mM D-glucose (Sigma), 1% N_2 supplement (Invitrogen, Merelbeke, Belgium), 100 U/ml penicillin (Cambrex), 100 U/ml streptomycin (Cambrex), 20 ng/ml of human recombinant epidermal growth factor (EGF, Sigma) and 20 ng/ml of recombinant human basic fibroblast growth factor (bFGF, R&D, Minneapolis, MN, USA). Cells were grown at 37°C in 5% CO_2 and 95% air with saturated humidity. They were passaged once cell clusters, with a diameter of about 100 μm , were formed, approximately 2 weeks after initial isolation. Subsequent passages were done every 1–2 weeks. When sufficiently large cell numbers were obtained, cells were further cultured in T75 flasks at a density of 750.000 per flask. After ten passages, cells were used for *in vitro* differentiation and *in vivo* transplantation experiments. At each passage NSC were evaluated for their GFP expression using flow cytometry analysis.

IN VITRO DIFFERENTIATION OF THE NEURAL STEM CELLS

Cells were differentiated by plating them at a density of 50000 cells/cm² on matrigel coated (BD biosciences, Erembodegem, Belgium) chamber slide systems (VWR International, Leuven, Belgium). During the first 24 hours of differentiation, cells were cultured in the presence of NSC growth medium with addition of 3% Fetal Calf Serum (FCS, Serum Supreme, Cambrex) which was devoid of EGF. This medium was then replaced by medium without EGF and bFGF, but with 3% FCS. Medium was changed every 48 hours for 5 days. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min and subjected to immunocytochemistry. The fixed cells were rinsed with 50 mM NH_4Cl for 10 min, permeabilized and blocked in phosphate-buffered saline (PBS) containing

0.4% fish skin gelatine (FSG) and 0,3% Triton X-100 (PBS/FSG/TX100). This was followed by incubation first with primary antibodies (ms, mouse monoclonal; rb, rabbit polyclonal): rb anti-tau 1:2000 (Dako, Heverlee, Belgium; A 0024), rb anti-GFAP 1:400 (Dako, Z0334), ms anti-RIP 1:5000 (Chemicon, MAB1580) and then with secondary antibodies Alexa Fluor 594 goat anti-rabbit IgG 1:1000 (Invitrogen, Merelbeke, Belgium; A11072) or Alexa Fluor 594 goat anti-mouse IgG 1:1000 (Invitrogen, A11020) diluted in PBS/FSG /TX100 for 2 hours each. Chamber slides were then rinsed and mounted with Vectashield mounting medium (Labconsult, Brussels, Belgium). Each chamber slide was stained for only one of the three markers. To assess the fraction of cells expressing a specific marker, immunopositive cells were counted in eight randomly selected high-power fields. The relative amount of cells positive for a specific marker was obtained by dividing the total number of immunopositive cells by the total number of counted nuclei. In control studies the primary antibody was omitted and no immunostaining was detected in these controls.

INTRAHIPPOCAMPAL KA INJECTIONS

Rats (Sprague Dawley, n=24, 175-200g, Harlan, the Netherlands) were anesthetized using isoflurane (Abbot, Louvain-La-Neuve, Belgium) and fixated in a stereotactic frame (Bilaney Consultants, Düsseldorf, Germany). Their skull was exposed and a hole was burred for injection of 0.2 µl KA solution (2 mg/ml) using a 30G injection needle attached to a Hamilton syringe (1µl). KA was injected in the right hippocampus (AP:-5.6, ML:+4.5 and DV:-5.5 mm relative to bregma) (Paxinos and Watson, 1998) over a time period of 1 minute. After injection the needle was left in place for an additional three minutes and then slowly retracted. The burr hole was closed with bonewax (Ethicon, Johnson and Johnson Medical, Dilbeek, Belgium) and the skin was closed. Gas anaesthetics were stopped and during the first minutes of awakening, the rat developed a status epilepticus (SE) which spontaneously stopped several hours after its induction.

TRANSPLANTATION OF NEURAL STEM CELLS

NSC were labelled by adding 5-bromo-2-deoxyuridine (BrdU, 5µM, Roche, Vilvoorde, Belgium) to the medium. After two days cells were harvested, mechanically dissociated and resuspended in medium consisting of Dulbecco's Phosphate Buffered Saline (DPBS; Invitrogen), 3 mM D-glucose (Sigma) and 20 ng/ml bFGF (R&D) in a concentration of 100,000 cells/µl. Transplantation of NSC was done in 3 experimental groups: in normal, non-lesioned rats (Norm group, n=12), in rats 3 days after KA injection (KA3d group, n=12) and in rats 3 weeks after KA injection (KA3w group, n=12). In both KA treated groups cells were transplanted at the same site where previously the lesion was made. Rats were anesthetized with a combination of ketamine and xylazine (80 mg/kg and 7.5 mg/kg respectively, IP). The animals were positioned into a stereotactic frame, their skull was exposed and a burr hole was made through which a transplantation needle (26S gauge) was lowered in the hippocampus (AP:-5.6, ML: +4.5 and DV:-5.5 mm relative to bregma). Cells were injected at a rate of 1 µl/min using a 10 µl Hamilton syringe as two deposits. After injecting 1µl of the cell suspension the needle was left in place for 2 minutes. The needle was then moved 0.5 mm up and an additional 0.4 µl was injected. The needle was not moved for an additional five minutes and was then slowly retracted. The burr hole was closed with

bonewax and the skin was closed. At the end of the transplantation procedure the viability of the remaining NSC was checked using the trypan blue exclusion method. Starting one day before transplantation, rats received daily injections (10 mg/kg, i.p.) of cyclosporin (Sandimmune, Novartis Pharma, Vilvoorde, Belgium) until they were killed (Pakzaban and others, 1995).

TISSUE PROCESSING

In all groups half of the rats (n=6) were killed three weeks after transplantation, the other half (n=6) six weeks after transplantation. Rats were terminally anesthetized using a pentobarbital overdose (180 mg/kg). The animals were then transcardially perfused with PBS at 37°C and postfixed overnight with 4% PFA. Brains were isolated and left overnight in 4% PFA at 4°C. Brain tissue was then cryoprotected in a 30% sucrose solution (30g/100ml) for three days. The cerebellum and frontal lobes were removed and the remaining brain tissue was frozen in ice-cold isopentane and stored in liquid nitrogen, until further processing.

IMMUNOHISTOCHEMICAL STAINING OF THE SECTIONS

Horizontal brain sections (30 µm) were made using a cryostat. Only sections containing hippocampus were preserved. Free-floating sections were washed 3 times in PBS. Then they were blocked for 45 minutes in PBS/FSG/TX100 followed by an overnight incubation with primary antibodies, also diluted in PBS/FSG/TX100: ms anti-NeuN 1:500 (Chemicon, MAB377), rb anti-GFAP 1:1000 (Dako, Z0334), ms anti-RIP 1:2000 (Chemicon, MAB1580), rb anti-NG2 1:200 (Biognost, Heule, Belgium; AB5320), rabbit serum anti-GFP (Invitrogen A6455), ms anti-BrdU 1:200 (Roche, 11170376001). This was followed by staining with secondary antibodies during 2 hours, also diluted in PBS/FSG /TX100: Alexa Fluor 594 goat anti-mouse IgG (H+L) 1:1000 (Invitrogen, A11020), Alexa Fluor 594 goat anti-rabbit IgG 1:1000 (Invitrogen, A11072) and FITC goat anti-rabbit IgG 1:1000 (Jackson, Suffolk, England; 111-096-047).

Before incubation with anti-BrdU antibody, a pre-treatment with formamide/SSC at 65°C for 2 hours, followed by incubation with respectively SSC (5 min, room temperature), 2N HCl (30 min, 37°C) and 0.1M boric acid (10 min, pH 8.5, room temperature) was necessary to denature the DNA. After incubation with the secondary antibody, sections were rinsed four times with PBS and nuclei were stained with 4'-6-diaminido-2-phenylindole (DAPI) during 1 minute. Afterwards sections were rinsed in PBS and mounted with Vectashield mounting medium. On the sections with BrdU-staining nuclei were not stained with DAPI. The sections were further analyzed with an epi-fluorescence microscope and a Bio-Rad Radiance 2100 confocal laser scanning microscope. Confocal images were processed with ImageJ (Rasband, W.S., U. S. National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij/>)

Epi-fluorescence microscopy was performed to assess the number of surviving, transplanted cells. To do this, only cells that double labelled for BrdU and GFP were counted. Because GFP fluorescence was lost due to pre-treatment of the tissue for BrdU immunostaining, we also used immunohistochemistry to identify GFP-containing cells. In preliminary experiments we found that there was no difference between the numbers of GFP containing cells before pre-treatment, identified by native GFP fluorescence, and after pre-treatment, identified by immunohistochemistry for GFP.

All GFP/BrdU double labelled cells on every third hippocampal section were counted. The Abercrombie formula for correction of cell counts was used to obtain estimates of total cell numbers from the raw cell counts (Abercrombie, 1946; Dobrossy and Dunnett, 2004):

$$P = (1/f) \times A \times [M / (D + M)]$$

where P is the corrected cell number, A is the raw cell count from the entire graft, f is the frequency of sections counted, M is the section thickness and D is the average diameter of the BrdU labelled nuclei. To compare cell survival between groups, numbers of cells were presented as fraction of the total number of transplanted cells.

Cell dispersion was measured on the same sections, used for assessment of survival, by determination of the maximal distance between two BrdU/GFP double-labelled cells for each section. Sections, containing only transplanted cells aligned along the needle tract, were excluded. The mean maximal distance was calculated and used as a measure for dispersion.

Every third slide containing transplanted cells was used for assessing colocalisation of GFP with neuronal (NeuN), astrocyte (GFAP) or oligodendrocyte (RIP, NG2) markers using confocal laser scanning microscopy. For each marker expression was checked in 20 GFP positive cells which were randomly selected from all sections stained for this specific marker.

DATA ANALYSIS

All values are expressed as means \pm standard error of the mean (SEM). Statistical analysis was performed using a two-factor (treatment and survival time) analysis of variance (ANOVA). If two-way ANOVA demonstrated a significant effect of treatment ($P < 0.05$) a Bonferroni post-hoc test was performed to determine which of the three treatment groups significantly differed.

RESULTS

IN VITRO CULTURE AND DIFFERENTIATION OF NSC

Neurosphere-forming NSC were successfully isolated from the SVZ of β -actin-GFP reporter mice. Two weeks after initial isolation, neurospheres, growing loosely attached to the substrate, were present in the culture. These neurospheres were passaged mechanically when their diameter ranged from 100 to 150 μ m. The dissociated cells were replated and gave rise to new neurospheres. These neurospheres were further passaged every 7 to 10 days after plating. Flow cytometry analysis, performed at each passage, showed that NSC stably expressed GFP for at least 10 passages (fig 1A).

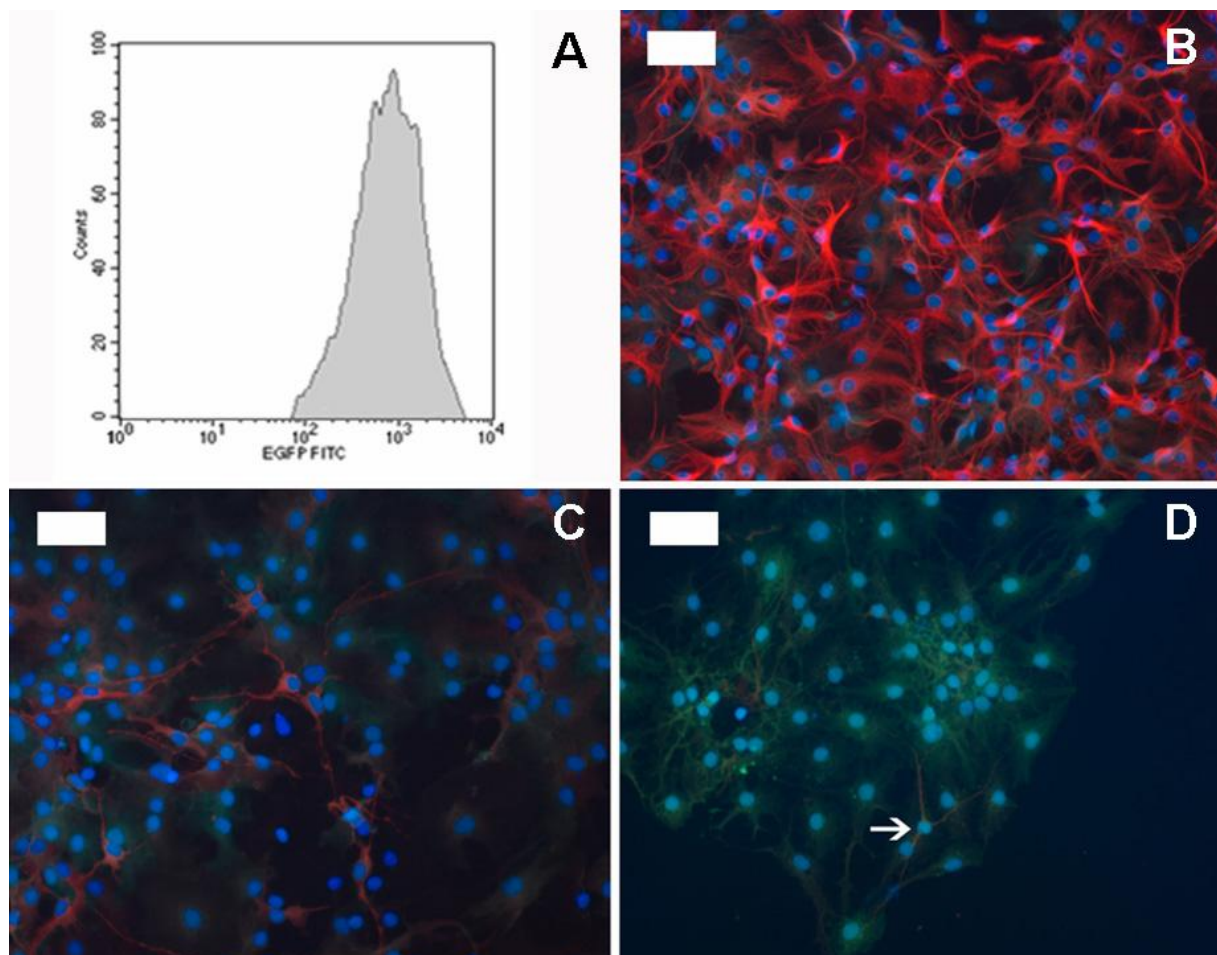


Fig 1: GFP expression and differentiation of NSC after ten passages *in vitro*. A: Flow cytometry analysis shows that NSC derived from β -actin-EGFP reporter mice still express GFP after 10 passages *in vitro*. (B-C): Plating these expanded NSC on matrigel coated plates in the absence of growth factors and the presence of 3% serum resulted in the differentiation towards neurons, astrocytes and oligodendrocytes. Five days after initiation of differentiation cells were stained for the presence of tau (neuronal marker), GFAP (astrocyte marker) and RIP (oligodendrocyte marker) using immunocytochemistry. B: The majority of the cells (37 %) expressed the glial marker GFAP. C: A large fraction of cells (14 %) differentiated towards neuronal cells expressing tau (middle). D: A very low fraction of cells (0.4%) expressed the oligodendrocyte marker RIP (right, arrow). About half of the cells were still immature and did not yet express differentiation markers. (Scale bar= 50 μ m)

After 10 passages a fraction of the cells was used for transplantation while the other fraction was subjected to *in vitro* differentiation. Quantitative analysis, five days after initiation of the differentiation, showed that the majority of the cells (36.9%) expressed the glial marker GFAP (fig 1B). A large fraction of cells (13.8 %) differentiated towards cells expressing the neuronal marker tau (fig 1C) and a very low fraction of cells (0.4 %) expressed the oligodendrocyte marker RIP (fig. 1D). About half of the cells did not express differentiation markers. All differentiated cells still expressed GFP after differentiation.

IN VIVO SURVIVAL AND DISPERSION OF NSC IN INTACT AND SCLEROTIC HIPPOCAMPUS

Three and six weeks after transplantation of 140,000 BrdU-labelled NSC in non-sclerotic (Norm group) and sclerotic hippocampus (KA3d and KA3w group), we checked for the presence of transplanted cells. We found that a high amount of cells stained positive for BrdU but not for GFP (fig. 2A). We counted only the number of cells that double-labelled for BrdU and GFP to assess survival to avoid overestimation of cell survival.

In non-sclerotic hippocampus the total number of BrdU/GFP expressing cells, three weeks after transplantation, was estimated to be ~1% of the total number of transplanted cells (Table 1). Comparing survival of transplanted cells using two-way ANOVA showed a significant effect ($p < 0.05$) of treatment. Post-hoc analysis could not reveal differences in survival between the Norm group and both KA groups indicating that the presence of the sclerosis did not significantly affect survival. We did find that survival of the NSC was significantly higher ($p < 0.05$) when grafted three days compared to three weeks after the lesion. Six weeks after transplantation survival in the KA3d group was even more than twice as much compared to survival in the KA3w group.

Table 1: Survival of GFP expressing NSC in non-sclerotic (Norm) and sclerotic (KA3d and KA 3w) hippocampus three and six weeks after transplantation.

	Total number of surviving cells			% of number of transplanted cells		
	Norm	KA3d	KA3w *	Norm	KA3d	KA3w *
three weeks	1460 ± 339	2484 ± 559	1394 ± 547	1.0 ± 0.2	1.4 ± 0.4	0.8 ± 0.3
six weeks	853 ± 282	2132 ± 286	632 ± 303	0.6 ± 0.2	1.2 ± 0.2	0.5 ± 0.2

NSC, isolated from the SVZ of β -actin-GFP reporter mice, were labelled with BrdU prior to transplantation. GFP/BrdU labelled cells were transplanted in non-sclerotic hippocampus (Norm group, n=12) and in sclerotic hippocampus three days (KA3d group, n=12) or three weeks (KA3w, n=12) after the KA lesion. Both three and six weeks after transplantation half of the transplanted animals were killed and the brain was sectioned. Each third hippocampal brain section was immunostained for BrdU and GFP and the number of double positive cells was counted on these sections. Using the formula of Abercrombie the total number of surviving GFP expressing cells in hippocampus was estimated. Results are expressed as mean ± SEM. * Indicates a significant ($p < 0.05$) lower survival of GFP expressing NSC in KA3w group compared to the KA3d group.

Two-way ANOVA did not demonstrate an effect of time although in all three groups the number of GFP expressing cells found six weeks after transplantation was lower compared to three weeks after transplantation.

Although we transplanted cells in two deposits (DV:-5.5 and – 5.0 mm relative to bregma), only one continuous cluster of transplanted cells could be identified. Since we made horizontal sections we only assessed dispersion in the horizontal plane. In the Norm group mean maximal distance between transplanted cells was about 1.3 mm three weeks after transplantation (Table 2, Norm). The presence of a sclerotic lesion clearly affected dispersion of the transplanted cells since the mean maximal distance between two cells in both KA groups was about 650 μ m, which was only half of the distance measured in non-sclerotic hippocampus. Two-way ANOVA confirmed that there was an effect of treatment ($p<0.05$) and post-hoc analysis showed that in both KA groups dispersion was significantly different from dispersion in the Norm group. ANOVA did not show an effect of time indicating that cells did not migrate three weeks after transplantation. Especially in non-sclerotic hippocampus, there was a tendency of the cells to align along fracture planes (the alvear layer, the hippocampal fissure and the subgranular layer of the dentate gyrus) of the hippocampus. In the sclerotic hippocampus BrdU/GFP positive cells tended to remain close to each other in dense clusters (fig. 2C). In all three groups, single or isolated groups of BrdU/GFP positive cells were found at the border of the lateral ventricles (fig 2D). In the Norm group, but not in both KA groups, isolated transplanted cells were found in the granule cell layer of the hippocampus (fig 2E).

Table 2: Dispersion of GFP expressing NSC in intact (Norm) and sclerotic (KA3d and KA 3w) hippocampus three and six weeks after transplantation.

Cell dispersion			
(Mean maximal distance measured on individual horizontal hippocampal sections)			
	Norm	KA3d *	KA3w *
three weeks	1250 \pm 264	646 \pm 58	639 \pm 148
six weeks	1392 \pm 384	751 \pm 113	514 \pm 237

NSC, isolated from the SVZ of β -actin-GFP reporter mice, were labelled with BrdU prior to transplantation. GFP/BrdU labelled cells were transplanted in non-sclerotic hippocampus (Norm group, n=12) and in sclerotic hippocampus three days (KA3d group, n=12) or three weeks (KA3w, n=12) after the KA lesion. Both three and six weeks after transplantation half of the transplanted animals were killed and the brain was sectioned. Each third hippocampal brain section was immunostained for BrdU and GFP and the maximal distance between two double positive cells was measured on these sections. The mean maximal distance was calculated and used as a measure for dispersion. Results are expressed as mean \pm SEM. * Indicates a significant ($p<0.05$) lower dispersion of GFP expressing NSC in both the KA3d and KA3w groups compared to the Norm group.

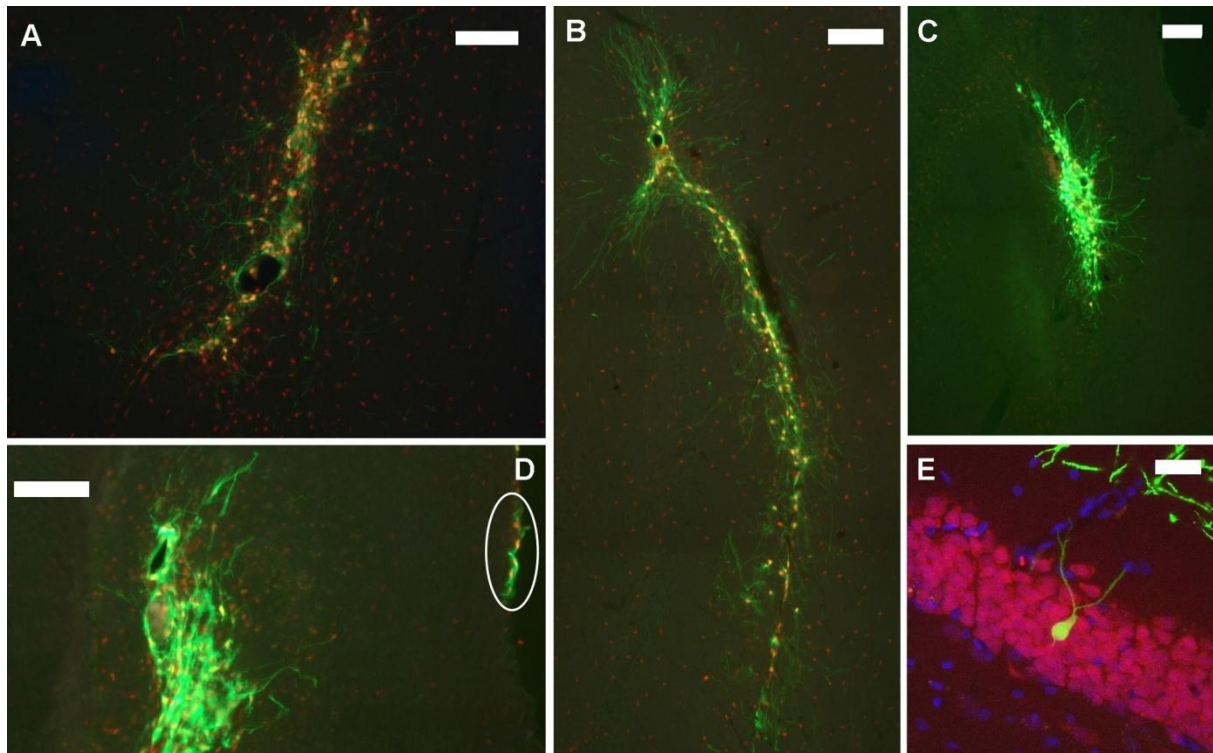


Fig 2: Survival and dispersion of transplanted BrdU/GFP positive NSC. Adult NSC, isolated from the SVZ of β -actin-GFP reporter mice, were labelled with BrdU prior to transplantation. GFP-BrdU labelled cells were transplanted in non-sclerotic hippocampus (Norm group, $n=12$) and in sclerotic hippocampus three days (KA3d group, $n=12$) or three weeks (KA3w, $n=12$) after the KA lesion. Three and six weeks after transplantation half of the transplanted animals were killed and the brain was sectioned. Each third hippocampal brain section was immunostained for BrdU and GFP. A: Immunostaining revealed a lot BrdU positive cells (red) that did not contain GFP (green). To assess survival only cells double-labelled for GFP and BrdU were counted. B: GFP-positive cells are spread over long distance and preferentially align along fracture planes in non-sclerotic hippocampus which is probably the result of passive diffusion due to cell injection. C: In sclerotic hippocampus transplanted cells stay in a rather dense cluster. D: Adult NSC are able to migrate towards the border of the lateral ventricle (circle) and E: to integrate into the granule cell layer (Scale bar is 100 μ m in A,B,C,D and 10 μ m in E)

IN VIVO DIFFERENTIATION OF NSC IN INTACT AND SCLEROTIC HIPPOCAMPUS

In all groups we found that a large percentage of the GFP positive cells double-labelled for the astrocyte marker, GFAP. Two-way ANOVA indicated an effect of treatment on the fraction of transplanted cells expressing GFAP and post-hoc analysis showed that there was a significant difference ($p<0.05$) between the Norm group and both KA groups. In the sclerotic hippocampus the fraction of transplanted cells expressing GFAP was significantly higher (about 50% to 70%) and almost double of the fraction in the non-sclerotic hippocampus (about 30% to 40%). Transplanted cells expressing GFAP in the Norm group had multiple fine processes, resembling normal astrocytes. In the KA groups GFAP expressing transplanted cells displayed dense staining of the enlarged cell bodies and enlarged cell processes, resembling reactive astrocytes (fig 3). Using ANOVA we could not demonstrate an effect of time on the number of GFAP expressing transplanted cells which indicates that all differentiation occurs in the first three weeks after transplantation.

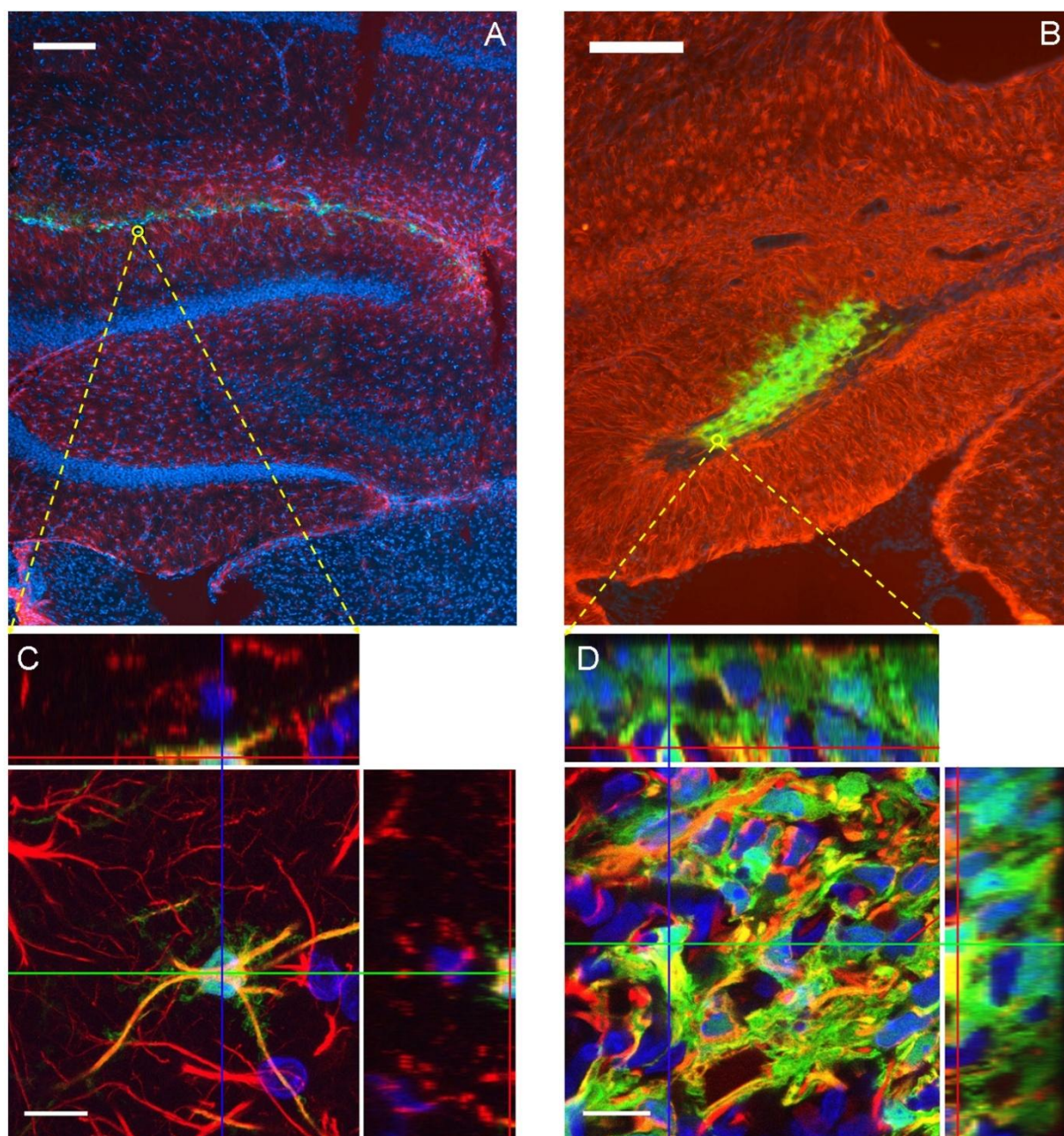


Fig 3: *In vivo* differentiation towards GFAP positive astrocytes. A and B: images, made using epi-fluorescence microscopy, show representative examples of sections containing GFP positive transplanted cells which also doublestain for GFAP (scale bar= 200 μ m). A: In non-sclerotic hippocampus NSC align along the hippocampal fissure where they predominantly differentiate towards GFAP positive astrocytes. B: In sclerotic hippocampus grafted cells remain in dense clusters and the majority of the cells express GFAP. C and D: confocal images showing the difference in morphology and staining pattern of GFAP expressing GFP positive, transplanted cells (scale bar=10 μ m). C: GFP positive cells, which express GFAP, form multiple thin filaments in a non-sclerotic hippocampus, resembling normal astrocytes. D: GFP positive cells, which express GFAP, show excessive staining for GFAP, an enlarged cell body and thick processes in a sclerotic hippocampus resembling reactive astrocytes.

We also evaluated the fraction of GFP positive cells expressing NeuN three and six weeks after transplantation in both non-sclerotic and sclerotic hippocampus. In all experimental groups a low percentage of cells differentiated towards neuronal cells (table 3). The largest fraction of neural differentiation, about 6%, was found in non-sclerotic hippocampus six weeks after transplantation. In non-sclerotic hippocampus, cells

expressing NeuN had migrated away from the transplanted cell cluster and were found integrated in the granule cell layer (GCL).

In sclerotic hippocampus NeuN expressing GFP positive cells did not integrate into the GCL but remained in close proximity of the cell cluster (fig 4). Using ANOVA we could not demonstrate effects of treatment nor time on the fraction of GFP positive cells expressing Even after an extensive search we could not identify GFP-positive cells that stained positive for one of both oligodendrocyte markers (RIP/NG2). A large proportion of grafted cells inside the cluster were not immunoreactive for any of the antigens tested.

Table 3: Proportion of GFP expressing NSC that demonstrated double immunolabelling for NeuN or GFAP in intact (Norm) and sclerotic (KA3d and KA 3w) hippocampus three and six weeks after transplantation.

	% of GFP+ cells expressing GFAP			% of GFP+ cells expressing NeuN		
	Norm	KA3d *	KA3w *	Norm	KA3d	KA3w
three weeks	30.0 ± 6.9	64.0 ± 7.6	50.1 ± 11.5	3.0 ± 2.0	2.0 ± 1.2	2.0 ± 1.2
six weeks	38.6 ± 6.1	69.0 ± 8.3	56.5 ± 7.3	5.8 ± 2.0	1.0 ± 1.0	3.8 ± 2.4

GFP labelled NSC, isolated from SVZ of β -actin-GFP reporter mice, were transplanted in non-sclerotic (Norm group, n=12) and in sclerotic hippocampus, lesioned with KA three days (KA3d group, n=12) or three weeks (KA3w, n=12) before grafting. Both three and six weeks after transplantation half of the transplanted animals were killed and the brain was sectioned. Every 15th hippocampal brain section was stained for GFAP or NeuN. In each animal, 20 GFP expressing cells were randomly selected from two sections stained for NeuN or GFAP. The fraction of GFP positive cells that immunostained for respectively the neuronal or the astrocyte marker was calculated. Results are expressed as mean \pm SEM. * Indicates a significant ($p < 0.05$) higher fraction of NSC expressing GFAP in both the KA3d and KA3w groups compared to the Norm group.

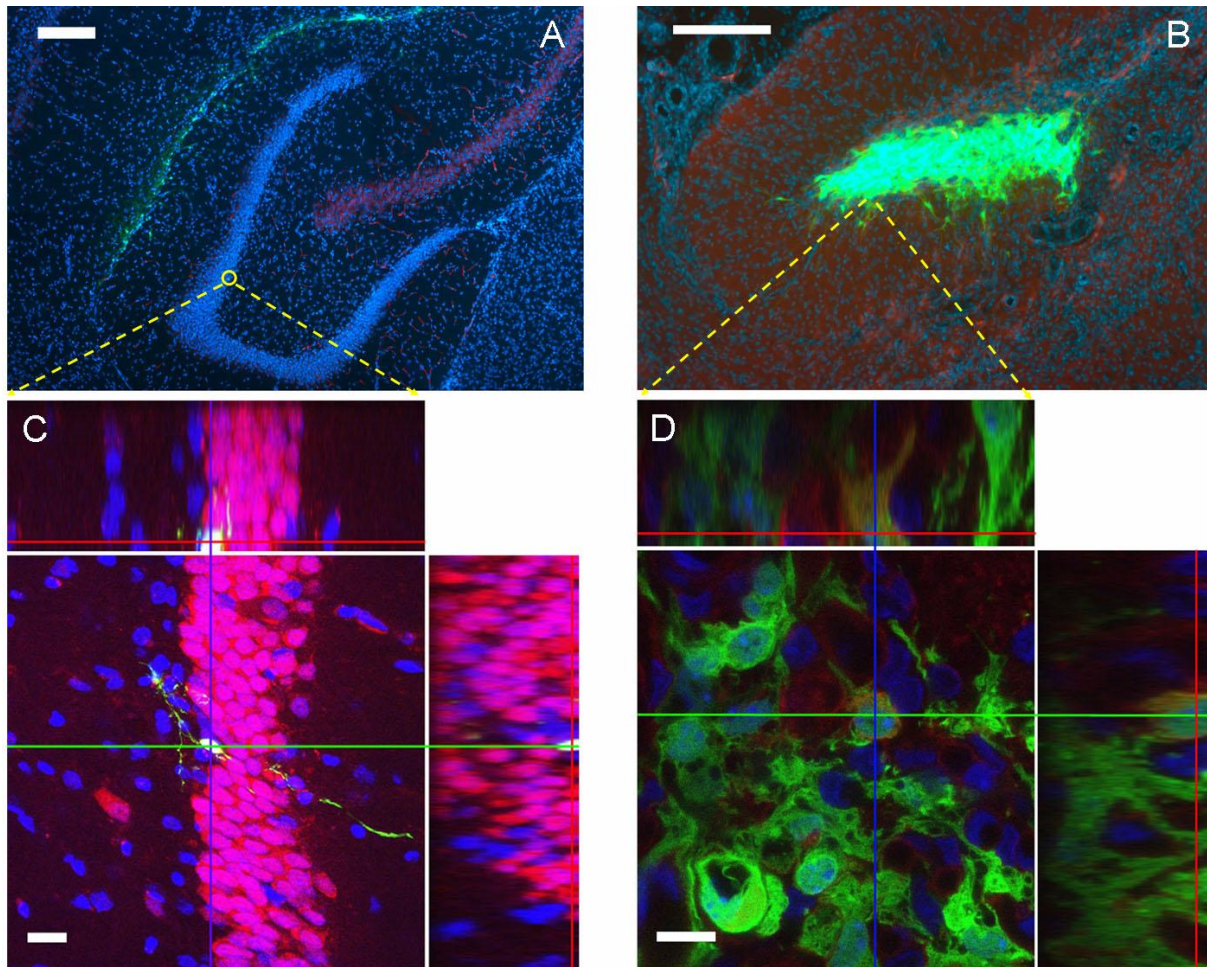


Fig 4: *In vivo* differentiation towards NeuN positive neuronal cells: A and B: images, made using epifluorescence microscopy, showing representative examples of sections containing GFP positive transplanted cells which also doublestain for NeuN (scale bar= 200 μ m). A: In non-sclerotic hippocampus some GFP positive cells were found in dentate granule cell layer and stained positive for NeuN, (yellow circle). B: In sclerotic hippocampus grafted cells did not integrate in granule cell layer but remained in dense clusters. Some of the cells within the cluster immunostained for NeuN. C and D: confocal images showing in closer detail NeuN expressing cells in non-sclerotic and sclerotic hippocampus (scale bar=10 μ m). C: In non-sclerotic hippocampus NeuN expressing cells are found integrated in the dentate granule layer. D: Sparse NeuN expressing GFP positive cells in a sclerotic hippocampus are found distributed throughout the cluster of transplanted cells.

DISCUSSION

The main findings of this study were the following: firstly, we found that a fraction of BrdU labelled NSC, isolated from the SVZ of adult β -actin-GFP reporter mice and expanded as neurospheres *in vitro*, was able to survive for at least six weeks after transplantation in non-sclerotic (Norm group) and sclerotic hippocampus (KA3d and KA3w group). Secondly, we found that a delay of three days between induction of the lesion and transplanting of the NSC results in a higher, more robust cell survival compared to a delay of three weeks. Thirdly, we showed that dispersion of transplanted cells was reduced in sclerotic hippocampus. Fourthly, we found that a large fraction of the transplanted NSC differentiated towards astrocytes and that this was enhanced by transplantation in a sclerotic hippocampus. Fifthly, we detected both in non-sclerotic and sclerotic hippocampus a small fraction of GFP expressing NSC that had differentiated towards cells expressing the neuronal marker, NeuN.

The number of GFP expressing cells that survived after transplantation for at least three weeks was about 1% of the total number of transplanted cells. This is rather low compared to other studies where a survival rate of 41% and 66% was found after transplantation of hippocampus-derived NSC in respectively the hippocampus of normal rats and the striatum of hemiparkinsonian rats (Gage and others, 1995; Dzieczapolski and others, 2003). In our study, we could have underestimated cell survival by excluding BrdU labelled cells that did not doublestain for GFP. Although presence of BrdU in GFP-negative cells could be explained by downregulation of GFP expression after transplantation, we had several reasons to be cautious about using only BrdU to assess cell survival. Firstly, we isolated the cells from transgenic mice in which stable expression of GFP is seen in all tissues, including brain, throughout life (Okabe and others, 1997; Manfra and others, 2001). Secondly, in this study the cells kept expressing GFP during *in vitro* culture and differentiation. Thirdly, in a recent study it was clearly shown that BrdU from dying cells can be recycled in endogenous proliferating cells, or cells undergoing simple DNA repair (Burns and others, 2006).

The survival of GFP expressing transplanted cells in our study more closely resembles the results of another recent transplantation study. In that study, SVZ-derived rat NSC, transfected with an adenoviral vector containing the gene encoding for GFP, were transplanted into an intact hippocampus of rats from the same or a different strain. These authors showed that after allogenic transplantation of 100,000 cells only 3000 GFP-expressing cells were retrieved three weeks after transplantation (Muraoka and others, 2006). This survival rate was higher if cell transplantation was done in an autologous manner. We have transplanted mouse cells into rat hosts. Although we used cyclosporine as immunosuppressant, interspecies barrier could be one of the contributing factors for the low survival.

In our study, we demonstrated that a longer delay between making the lesion and performing the transplantation resulted into lower survival rates. This time dependant decrease in survival was also observed after transplantation of fetal CA3 neurons in a KA induced lesion. In these experiments survival of cells, transplanted 45 days after making the lesion, was up to four times lower compared to transplantation four

days after the lesion (Zaman and others, 2001; Turner and Shetty, 2003). This time dependant effect on survival of transplanted cells was explained by the presence of an enhanced neurotrophic factor environment for up to 10 to 14 days after lesion development (Lowenstein and others, 1993). A combined neurotrophic supplementation and caspase inhibition has been shown to dramatically enhance survival of grafts placed into the injured adult hippocampus at an extended time-point (i.e. 4-months) after injury (Hattiangady and others, 2006). This may represent an alternative strategy to enhance survival of SVZ-derived NSC in our setup.

The majority of the GFP positive cells in non-sclerotic hippocampus were found to align fracture planes of the hippocampus. In sclerotic hippocampus, dispersion of transplanted cells was lower and cells remained in dense clusters. This could be due to the presence of the glial scar which formed a barrier which transplanted cells could not cross. An alternative explanation could be that cells remained close to the lesion because they were attracted to it. Endogenous SVZ-derived NSC possess a high pathotropism and have been shown to be strongly attracted to excitotoxic lesions in hippocampal CA1 and CA3 regions, induced by SE. The NSC migrated towards the lesions where they differentiated towards glial cells (Parent and others, 2006). Although our study did not allow discriminating whether dispersion of transplanted cells was the result of passive diffusion or active migration, we found isolated cells at a distance of the injection site at places that could only be reached by active migration through the parenchyma, for example in the GCL or at the border of the lateral ventricles.

Doublestaining for neuronal, astrocyte and oligodendrocyte markers showed that the majority of the NSC differentiated towards astrocytes. Several other studies have shown that a high percentage of expanded NSC adopt a glial fate upon transplantation into the adult brain (Gage and others, 1995; Carpenter and others, 1997; Hammang and others, 1997; Dziewczapolski and others, 2003; Shear and others, 2004; Soares and Sotelo, 2004; Muraoka and others, 2006). The type of glial fate seems to depend on the site of transplantation. When hippocampus-derived NSC were transplanted in an intact hippocampus, a large cell fraction differentiated towards astrocytes (Gage and others, 1995). When these cells were transplanted into the striatum, the majority generated NG2-positive oligodendrocyte precursors while only 0.1% of the cells differentiated towards astrocytes (Dziewczapolski and others, 2003). Based on two findings in our study we hypothesize that the transplanted NSC were recruited in hippocampal sclerosis in response to the KA lesion. Firstly, we showed that transplantation in sclerotic hippocampus resulted in a higher fraction of transplanted cells expressing GFAP. Secondly, in lesioned hippocampus, GFAP positive cells showed intense staining for GFAP, enlarged cell bodies and thickened processes and therefore resembled reactive astrocytes.

Next to this glial differentiation a portion of NSC differentiated towards cells expressing NeuN, a marker for mature neurons. Other transplantation studies have indicated that NSC, derived from adult brain, only generate neurons in the adult brain when they are transplanted into neurogenic regions such as the hippocampus or the olfactory bulb (Gage and others, 1995; Suhonen and others, 1996; Shihabuddin and others, 2000; Lie and others, 2002; Richardson and others, 2005b; Muraoka and others, 2006). Adult NSC even respond to local differentiation cues and are able to adopt region specific neural phenotypes (Shihabuddin and others, 2000; Richardson and others, 2005b). The maximal fraction of 6% NeuN expressing cells, six weeks after

transplantation in non-sclerotic hippocampus, resembled the fraction seen in a similar study performed by Muraoka and coworkers (Muraoka and others, 2006). These authors showed that the number of NeuN expressing could be doubled by performing autologous transplantation. In another study the fraction of SVZ-derived NSC differentiated towards neurons was even higher (about 27%). In this study NSC were cultured as monolayer instead of neurospheres, which could promote the selection of neuroprogenitor cells (Richardson and others, 2005b). Also these authors used BrdU as the only marker to identify transplanted cells. Because the hippocampus is a region containing a lot of dividing neuronal precursors, a transfer of BrdU from dying transplanted cells to dividing endogenous neuroblasts is possible, resulting in an overestimation of neuronal differentiation (Burns and others, 2006). Comparing neuronal differentiation between groups in our study revealed that the presence of a KA lesion did not affect the fraction of NSC differentiating towards neurons but did affect the site of neural differentiation. In non-lesioned hippocampus transplanted cells expressing NeuN were found exclusively in the granule cell layer while in the lesioned hippocampus NeuN expressing cells were found on different locations in the hippocampus. This could be explained by the lack of signals driving migration and integration into the GCL.

In conclusion, our study is the first in which NSC were transplanted into a KA lesioned hippocampus. By comparing this to transplantation of NSC in non-lesioned hippocampus we showed that a comparable but limited fraction of NSC is able to survive for at least six weeks after transplantation and that transplanted NSC seem to contribute to hippocampal sclerosis in response to the KA lesion. Further studies are needed to look for manipulations that could enhance the fraction of cells that survive after transplantation (e.g. autologous transplantation, pre-treatment with anti-apoptotic substances, local infusion of survival factors). However based on their stable survival, NSC may be investigated to deliver seizure modifying substances in the sclerotic hippocampus.

REFERENCES

- Abercrombie M. 1946. Estimation of nuclear population from microtome sections. *The Anatomical Record* 94(2):239-47.
- Bengzon J, Kokaia M, Brundin P, Lindvall O. 1990. Seizure suppression in kindling epilepsy by intrahippocampal locus coeruleus grafts: evidence for an alpha-2-adrenoreceptor mediated mechanism. *Exp Brain Res* 81(2):433-7.
- Bjorklund A, Lindvall O. 2000. Cell replacement therapies for central nervous system disorders. *Nature Neuroscience* 3(6):537-44.
- Bortolotto ZA, Calderazzo L, Cavalheiro EA. 1990. Some evidence that intrahippocampal grafting of noradrenergic neurons suppresses spontaneous seizures in epileptic rats. *Braz J Med Biol Res* 23(12):1267-9.
- Burns TC, Ortiz-Gonzalez XR, Gutierrez-Perez M, Keene CD, Sharda R, Demorest ZL, Jiang Y, Nelson-Holte M, Soriano M, Nakagawa Y, Luquin MR, Garcia-Verdugo JM, Prosper F, Low WC, Verfaillie CM. 2006. Thymidine Analogs Are Transferred from Prelabeled Donor to Host Cells in the Central Nervous System After Transplantation: A Word of Caution. *Stem Cells* 24(4):1121-7.
- Carpenter MK, Winkler C, Fricker R, Emerich DF, Wong SC, Greco C, Chen E-Y, Chu Y, Kordower JH, Messing A. 1997. Generation and Transplantation of EGF-Responsive Neural Stem Cells Derived from GFAP-hNGF Transgenic Mice. *Experimental Neurology* 148(1):187-204.
- Clusmann H, Schramm J, Kral T, Helmstaedter C, Ostertun B, Fimmers R, Haun D, Elger CE. 2002. Prognostic factors and outcome after different types of resection for temporal lobe epilepsy. *J Neurosurg* 97(5):1131-41.
- Dobrossy MD, Dunnett SB. 2004. Environmental enrichment affects striatal graft morphology and functional recovery. *European Journal of Neuroscience* 19(1):159-68.
- Dziewczapolski G, Lie DC, Ray J, Gage FH, Shults CW. 2003. Survival and differentiation of adult rat-derived neural progenitor cells transplanted to the striatum of hemiparkinsonian rats. *Exp Neurol* 183(2):653-64.
- Engel J, Jr. 2001. Mesial Temporal Lobe Epilepsy: What Have We Learned? *Neuroscientist* 7(4):340-52.
- Fedele DE, Koch P, Scheurer L, Simpson EM, Mohler H, Brustle O, Boison D. 2004. Engineering embryonic stem cell derived glia for adenosine delivery. *Neurosci Lett* 370(2-3):160-5.
- Ferencz I, Kokaia M, Elmer E, Keep M, Kokaia Z, Lindvall O. 1998. Suppression of kindling epileptogenesis in rats by intrahippocampal cholinergic grafts. *European Journal of Neuroscience* 10(1):213-20.
- Ferencz I, Leanza G, Nanobashvili A, Kokaia Z, Kokaia M, Lindvall O. 2001. Septal cholinergic neurons suppress seizure development in hippocampal kindling in rats: comparison with noradrenergic neurons. *Neuroscience* 102(4):819-32.
- Freeman JM, Vining EP, Pillas DJ, Pyzik PL, Casey JC, Kelly L, and Millicent. 1998. The Efficacy of the Ketogenic Diet---1998: A Prospective Evaluation of Intervention in 150áChildren. *Pediatrics* 102(6):1358-63.
- Gage FH. 2000. Mammalian neural stem cells. *Science* 287(5457):1433-8.
- Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, Peterson DA, Suhr ST, Ray J. 1995. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci U S A* 92(25):11879-83.
- Gernert M, Thompson KW, Loscher W, Tobin AJ. 2002. Genetically engineered GABA-producing cells demonstrate anticonvulsant effects and long-term transgene expression when transplanted into the central piriform cortex of rats. *Exp Neurol* 176(1):183-92.
- Gobbel GT, Choi SJ, Beier S, Niranjan A. 2003. Long-term cultivation of multipotential neural stem cells from adult rat subependyma. *Brain Res* 980(2):221-32.
- Gritti A, Frolichsthal-Schoeller P, Galli R, Parati EA, Cova L, Pagano SF, Bjornson CR, Vescovi AL. 1999. Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. *J Neurosci* 19(9):3287-97.

- Guttinger M, Fedele D, Koch P, Padrun V, Pralong WF, Brustle O, Boison D. 2005a. Suppression of kindled seizures by paracrine adenosine release from stem cell-derived brain implants. *Epilepsia* 46(8):1162-9.
- Guttinger M, Padrun V, Pralong WF, Boison D. 2005b. Seizure suppression and lack of adenosine A1 receptor desensitization after focal long-term delivery of adenosine by encapsulated myoblasts. *Exp Neurol* 193(1):53-64.
- Hamang JP, Archer DR, Duncan ID. 1997. Myelination Following Transplantation of EGF-Responsive Neural Stem Cells into a Myelin-Deficient Environment. *Experimental Neurology* 147(1):84-95.
- Handforth A, DeGiorgio CM, Schachter SC, Uthman BM, Naritoku DK, Tecoma ES, Henry TR, Collins SD, Vaughn BV, Gilmartin RC, Labar DR, Morris GL, III, Salinsky MC, Osorio I, Ristanovic RK, Labiner DM, Jones JC, Murphy JV, Ney GC, Wheless JW. 1998. Vagus nerve stimulation therapy for partial-onset seizures: a randomized active-control trial. *Neurology* 51(1):48-55.
- Hattiangady B, Rao MS, Zaman V, Shetty AK. 2006. Incorporation of embryonic CA3 cell grafts into the adult hippocampus at 4-months after injury: Effects of combined neurotrophic supplementation and caspase inhibition. *Neuroscience* 139(4):1369-83.
- Herrera DG, Garcia-Verdugo JM, Alvarez-Buylla A. 1999. Adult-derived neural precursors transplanted into multiple regions in the adult brain. *Ann Neurol* 46(6):867-77.
- Huber A, Padrun V, Deglon N, Aebischer P, Mohler H, Boison D. 2001. Grafts of adenosine-releasing cells suppress seizures in kindling epilepsy. *Proc Natl Acad Sci U S A* 98(13):7611-6.
- Kelly S, Bliss TM, Shah AK, Sun GH, Ma M, Foo WC, Masel J, Yenari MA, Weissman IL, Uchida N, Palmer T, Steinberg GK. 2004. Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. *PNAS* 101(32):11839-44.
- Kokaia M, Cenci MA, Elmer E, Nilsson OG, Kokaia Z, Bengzon J, Bjorklund A, Lindvall O. 1994. Seizure development and noradrenaline release in kindling epilepsy after noradrenergic reinnervation of the subcortically deafferented hippocampus by superior cervical ganglion or fetal locus coeruleus grafts. *Exp Neurol* 130(2):351-61.
- Kwan P, Brodie MJ. 2000. Early identification of refractory epilepsy. *N Engl J Med* 342(5):314-9.
- Lie DC, Dzieczapolski G, Willhoite AR, Kaspar BK, Shults CW, Gage FH. 2002. The adult substantia nigra contains progenitor cells with neurogenic potential. *J Neurosci* 22(15):6639-49.
- Lindvall O, Bengzon J, Brundin P, Kalen P, Kokaia M. 1990. Locus coeruleus grafts in hippocampal kindling epilepsy: noradrenaline release, receptor specificity and influence on seizure development. *Prog Brain Res* 82:339-46.
- Lois C, Alvarez-Buylla A. 1993. Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc Natl Acad Sci U S A* 90(5):2074-7.
- Loscher W, Ebert U, Lehmann H, Rosenthal C, Nikkhah G. 1998. Seizure suppression in kindling epilepsy by grafts of fetal GABAergic neurons in rat substantia nigra. *J Neurosci Res* 51(2):196-209.
- Lowenstein DH, Seren MS, Longo FM. 1993. Prolonged increases in neurotrophic activity associated with kainate-induced hippocampal synaptic reorganization. *Neuroscience* 56(3):597-604.
- Manfra DJ, Chen SC, Yang TY, Sullivan L, Wiekowski MT, Abbondanzo S, Vassileva G, Zalamea P, Cook DN, Lira SA. 2001. Leukocytes Expressing Green Fluorescent Protein as Novel Reagents for Adoptive Cell Transfer and Bone Marrow Transplantation Studies. *Am J Pathol* 158(1):41-7.
- Muraoka K, Shingo T, Yasuhara T, Kameda M, Yuan W, Hayase H, Matsui T, Miyoshi Y, Date I. 2006. The high integration and differentiation potential of autologous neural stem cell transplantation compared with allogeneic transplantation in adult rat hippocampus. *Exp Neurol* 199(2):311-27.
- Nilsen KE, Cock HR. 2004. Focal treatment for refractory epilepsy: hope for the future? *Brain Res Brain Res Rev* 44(2-3):141-53.
- Nunes MC, Roy NS, Keyoung HM, Goodman RR, McKhann G2, Jiang L, Kang J, Nedergaard M, Goldman SA. 2003. Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. *Nat Med* 9(4):439-47.

- Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 1997. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* 407(3):313-9.
- Pakzaban P, Deacon TW, Burns LH, Dinsmore J, Isacson O. 1995. A novel mode of immunoprotection of neural xenotransplants: Masking of donor major histocompatibility complex class I enhances transplant survival in the central nervous system. *Neuroscience* 65(4):983-96.
- Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH. 1999. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J Neurosci* 19(19):8487-97.
- Palmer TD, Ray J, Gage FH. 1995. FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Mol Cell Neurosci* 6(5):474-86.
- Parent JM, von dem BN, Lowenstein DH. 2006. Prolonged seizures recruit caudal subventricular zone glial progenitors into the injured hippocampus. *Hippocampus* 16(3):321-8.
- Paxinos G, Watson C. 1998. *The Rat Brain in stereotaxic coordinates*. Fourth Edition ed. San Diego: Academic Press.
- Raedt R, De Smedt T, Van Melkebeke D, Claeys P, Van Dycke A, Wyckhuys T, Vonck K, Wadman W, Boon P. 2007. Seizures in the intrahippocampal kainic acid epilepsy model: characterization using long term video-EEG monitoring in the rat. *Acta Neurologica Scandinavica* submitted.
- Reynolds BA, Weiss S. 1996. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* 175(1):1-13.
- Richardson RM, Broaddus WC, Holloway KL, Fillmore HL. 2005a. Grafts of adult subependymal zone neuronal progenitor cells rescue hemiparkinsonian behavioral decline. *Brain Research* 1032(1-2):11-22.
- Richardson RM, Broaddus WC, Holloway KL, Sun D, Bullock MR, Fillmore HL. 2005b. Heterotypic neuronal differentiation of adult subependymal zone neuronal progenitor cells transplanted to the adult hippocampus. *Molecular and Cellular Neuroscience* 28(4):674-82.
- Ross KC, Waldman BC, Conejero-Goldberg C, Freed W, Coleman JR. 2002. Transplantation of M213-20 cells with enhanced GAD67 expression into the inferior colliculus alters audiogenic seizures. *Exp Neurol* 177(1):338-40.
- Shear DA, Tate MC, Archer DR, Hoffman SW, Hulce VD, LaPlaca MC, Stein DG. 2004. Neural progenitor cell transplants promote long-term functional recovery after traumatic brain injury. *Brain Research* 1026(1):11-22.
- Shihabuddin LS, Horner PJ, Ray J, Gage FH. 2000. Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. *J Neurosci* 20(23):8727-35.
- Shihabuddin LS, Ray J, Gage FH. 1997. FGF-2 is sufficient to isolate progenitors found in the adult mammalian spinal cord. *Exp Neurol* 148(2):577-86.
- Soares S, Sotelo C. 2004. Adult neural stem cells from the mouse subventricular zone are limited in migratory ability compared to progenitor cells of similar origin. *Neuroscience* 128(4):807-17.
- Spencer SS. 2002. When should temporal-lobe epilepsy be treated surgically? *The Lancet Neurology* 1(6):375-82.
- Suhonen JO, Peterson DA, Ray J, Gage FH. 1996. Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. *Nature* 383(6601):624-7.
- Svendsen CN, Caldwell MA, Ostenfeld T. 1999. Human neural stem cells: isolation, expansion and transplantation. *Brain Pathol* 9(3):499-513.
- Thompson K, Anantharam V, Behrstock S, Bongarzone E, Campagnoni A, Tobin AJ. 2000. Conditionally immortalized cell lines, engineered to produce and release GABA, modulate the development of behavioral seizures. *Exp Neurol* 161(2):481-9.
- Thompson KW. 2005. Genetically engineered cells with regulatable GABA production can affect afterdischarges and behavioral seizures after transplantation into the dentate gyrus. *Neuroscience* 133(4):1029-37.
- Thompson KW, Suchomelova LM. 2004. Transplants of cells engineered to produce GABA suppress spontaneous seizures. *Epilepsia* 45(1):4-12.

- Turner DA, Shetty AK. 2003. *Clinical prospects for neural grafting therapy for hippocampal lesions and epilepsy. Neurosurgery* 52(3):632-44.
- Velasco F, Carrillo-Ruiz JD, Brito F, Velasco M, Velasco AL, Marquez I, Davis R. 2005. *Double-blind, randomized controlled pilot study of bilateral cerebellar stimulation for treatment of intractable motor seizures. Epilepsia* 46(7):1071-81.
- Vescovi AL, Parati EA, Gritti A, Poulin P, Ferrario M, Wanke E, Frolichsthal-Schoeller P, Cova L, Arcellana-Panlilio M, Colombo A, Galli R. 1999. *Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. Exp Neurol* 156(1):71-83.
- Vonck K, Boon P, Achten E, De RJ, Caemaert J. 2002. *Long-term amygdalohippocampal stimulation for refractory temporal lobe epilepsy. Ann Neurol* 52(5):556-65.
- Weiss S, Dunne C, Hewson J, Wohl C, Wheatley M, Peterson AC, Reynolds BA. 1996. *Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. J Neurosci* 16(23):7599-609.
- Westerlund U, Svensson M, Moe MC, Varghese M, Gustavsson B, Wallstedt L, Berg-Johnsen J, Langmoen IA. 2005. *Endoscopically harvested stem cells: a putative method in future autotransplantation. Neurosurgery* 57(4):779-84.
- Wiebe S, Blume WT, Girvin JP, Eliasziw M. 2001. *A randomized, controlled trial of surgery for temporal-lobe epilepsy. N Engl J Med* 345(5):311-8.
- Zaman V, Turner DA, Shetty AK. 2001. *Prolonged postlesion transplantation delay adversely influences survival of both homotopic and heterotopic fetal hippocampal cell grafts in kainate-lesioned CA3 region of adult hippocampus. Cell Transplant* 10(1):41-52.

Chapter 6

Modulation of endogenous neurogenesis in TLE

MANUSCRIPT 6

RAEDT R., BOON P., PERSSON A., ALBORN A.M., BOTERBERG T., VAN DYCKE A., LINDER B., DE SMEDT T., WADMAN W., BEN-MENACHEM E., ERIKSSON P.S.

Radiation of the rat brain suppresses seizure-induced neurogenesis and transiently enhances excitability during kindling acquisition.

Epilepsia. June 6 2007, OnlineEarly article ([doi:10.1111/j.1528-1167.2007.01146.x](https://doi.org/10.1111/j.1528-1167.2007.01146.x))

Radiation of the Rat Brain Suppresses Seizure-Induced Neurogenesis and Transiently Enhances Excitability during Kindling Acquisition

*Robrecht Raedt, *Paul Boon, †Asa Persson, †Ann-Marie Alborn, ‡Tom Boterberg,
*Annelies Van Dycke, †Birgit Linder, *Tim De Smedt, §Wytse J. Wadman,
†Elinor Ben-Menachem, and †Peter S. Eriksson

**Laboratory for Clinical and Experimental Neurophysiology, Department of Neurology, Ghent University Hospital, Ghent, Belgium;
†Centre for Brain Repair and Rehabilitation (CBR), Institute of Neuroscience and Physiology, Sahlgrenska Academy,
Göteborg University, Göteborg, Sweden; ‡Department of Radiotherapy, Ghent University Hospital, Ghent, Belgium; and §Centre for
Neuroscience, Swammerdam Institute of Life Sciences University of Amsterdam, Amsterdam, The Netherlands*

In this manuscript we describe an experiment to further elucidate the role of neurogenesis in epileptogenesis. We subjected rats to low-dose radiation, in order to suppress hippocampal neurogenesis, and evaluated the effect of radiation on the development of rapid hippocampal kindling. We radiated the whole brain of half of rats using a small dose of 8 Gy. Both non-radiated and radiated rats were subjected to a rapid hippocampal kindling protocol one day after radiation. Suppression of seizure-induced neurogenesis was evaluated during the kindling acquisition (first week after radiation) and the kindling retest phase (two and three weeks after radiation). We assessed the effect of radiation on afterdischarge threshold, afterdischarge duration and kindling rate both during kindling acquisition and the kindling retest phase.

SUMMARY

Adult hippocampal neurogenesis is enhanced in several models for temporal lobe epilepsy (TLE). In this study we used low-dose, whole brain radiation to suppress hippocampal neurogenesis and then studied the effect of this treatment on epileptogenesis in a kindling model for TLE.

Half of the rats were exposed to a radiation dose of 8 Gy one day before the initiation of a rapid kindling protocol. Afterdischarge threshold (ADT), afterdischarge duration (ADD), clinical seizure severity and inflammation were compared between groups. On the first and third day after radiation, rats were injected with 5'-bromo-2'-deoxyuridine (BrdU) to evaluate neurogenesis. Seven and twenty-one days after radiation, numbers of doublecortin (DCX) positive neuroblasts in subgranular zone and granule cell layer were compared between groups.

In this study, we showed that radiation significantly suppressed neurogenesis and neuroblast production during kindling acquisition. Radiation also prevented an increase in ADT which became significantly lower in radiated rats. On the third and fourth kindling acquisition day radiated rats developed more severe seizures more rapidly, which resulted in a significantly higher mean severity score on these days. Differences in ADD could not be demonstrated.

Our results demonstrate that brain radiation with a relatively low dose effectively suppressed the generation of new granule cells and transiently enhanced excitability during kindling acquisition. Although seizure-induced neurogenesis was lower in the radiated rats we could not detect a strong effect on the final establishment of the permanent fully kindled state, which argues against a prominent role of seizure-induced neurogenesis in epileptogenesis.

INTRODUCTION

Recent studies have demonstrated that active neurogenesis takes place in discrete regions of the adult brain in mammals, including humans (Kempermann and Gage, 1999; Gross, 2000; Lie and others, 2004; Ming and Song, 2005). Brain regions where neurogenesis is most prominent are the subventricular zone (SVZ) of the lateral ventricle and both the subgranular zone (SGZ) and granule cell layer (GCL) of the hippocampus (Eriksson and others, 1998; Alvarez-Buylla and others, 2000; van Praag and others, 2002). Outside these regions, neurogenesis is either very sparse or non-existent in the intact adult brain. Neurogenesis is a dynamic process consisting of different subsequent stages, including proliferation, fate specification, migration, synaptic integration and survival.

In the dentate gyrus a subset of astrocytes, residing in the SGZ, has been proposed to be the neural stem cells. These cells divide and give rise to neuroblasts, which exit the cell cycle and migrate into the GCL where they further develop to mature granule cells, establish synaptic connectivity and become functionally integrated (Kempermann and others, 2004).

Experimentally induced seizures can modulate all stages of neurogenesis in dentate gyrus. Status epilepticus (SE) as well as kindling have stimulating effects on the proliferation of neural stem and/or precursor cells in the SGZ resulting in a net increase in granule cell neurogenesis (Parent and others, 1997; Bengzon and others, 1997; Gray and Sundstrom, 1998; Parent and others, 1998; Scott and others, 1998; Nakagawa and others, 2000; Ferland and others, 2002; Romcy-Pereira and Garcia-Cairasco, 2003; Huttman and others, 2003; Mohapel and others, 2004; Jessberger and others, 2005; Smith and others, 2005; Parent and others, 2006). In the chemoconvulsant and electrogenic status epilepticus models, neurogenesis is increased five- to tenfold after a latent period of a few days (Parent and others, 1997; Gray and Sundstrom, 1998; Jessberger and others, 2005). In the kindling model, neurogenesis is increased respectively three to six times (Bengzon and others, 1997; Smith and others, 2005). This increase in neurogenesis is only temporary and returns back to baseline levels or even below in the first month after seizures (Parent and others, 1997; Nakagawa and others, 2000).

In addition to an enhanced neurogenesis, seizures can also affect migration of the neuroblasts. In both the amygdala kindling model and SE models newly generated neurons were found in ectopic locations in the hilus (Parent and others, 1997; Scharfman and others, 2000; Smith and others, 2005; Pierce and others, 2005; Shapiro and others, 2005; Shapiro and Ribak, 2005; Parent and others, 2006; Shapiro and Ribak, 2006; Bonde and others, 2006). Studies in the PILO SE model show that these ectopic neurons have morphological and electrophysiological properties comparable with granule cells in the GCL (Scharfman and others, 2000). However when these cells are examined a few months after status, they display spontaneous, regular epileptiform bursts synchronously or a few milliseconds later than epileptiform bursts in CA3 cells (Scharfman and others, 2000). Synaptic integration of newborn neurons can be altered in an epileptic situation. Seizures can accelerate the development of excitatory input to immature dentate granule cells during the first two weeks after their formation (Overstreet-Wadiche and others, 2006). Seizure-activity also seems to induce part

of the newborn neurons to form aberrant mossy fiber recurrent connections and persistent basal dendrites which project into the hilus and are innervated by mossy fibers (Parent and others, 1997; Pierce and others, 2005; Shapiro and Ribak, 2005; Shapiro and Ribak, 2006).

The kindling model is a widely accepted experimental model for TLE (Goddard, 1983; McNamara, 1984). Regular electrical kindling consists of daily stimulating a discrete area of the limbic system for one second, every day. Every stimulus triggers an electrographic event, the afterdischarge (AD), whose threshold (the intensity of the current needed to evoke an AD) and duration reflect the level of excitability within the limbic circuit (McNamara, 1984). As the electrographic response to the kindling stimulation intensifies, behavioural responses to the stimulation increase in severity. An animal is considered to be fully kindled if kindling stimulation reproducibly evokes a generalized tonic-clonic seizure. Once an animal is fully kindled it remains displaying stage 5 seizures, in response to stimulation, for months or years (Goddard and others, 1969). Using the regular kindling protocol, it takes 10 to 15 days for the rodent to become fully kindled, strongly dependent on the region which is stimulated. There are also alternative kindling protocols where rodents can be fully kindled over a shorter period. The alternate day rapid hippocampal kindling protocol allows to fully kindle a rodent with 48 stimulations, spread over the course of four stimulation days (Lothman and others, 1985; Lothman and Williamson, 1994). In case of hippocampal kindling, neurogenesis is already increased three-fold five days after experiencing one single seizure and six-fold after 40 kindling stimulations (Bengzon and others, 1997). Whether this seizure-induced neurogenesis contributes to kindling epileptogenesis and the generation of a permanent fully kindled state is not yet elucidated.

Therefore, in this study, we exposed rats one day before rapid hippocampal kindling to low-dose whole brain radiation, a commonly used technique to suppress neurogenesis (Wojtowicz, 2006). Studies on the mechanism of radiation-induced suppression of neurogenesis show that radiation causes an increase in apoptosis of neural precursor cells and early postmitotic neurons together with a decrease in neural stem/precursor cells proliferation (Peissner and others, 1999; Monje and others, 2002; Monje and Palmer, 2003). Low dose (1-15 Gy) radiation does not cause gross tissue changes and spares adult neurons (Calvo and others, 1988; Tada and others, 2000). However, the use of this radiation also has limitations since other effects of radiation, besides suppression of neurogenesis, such as inflammation, alterations in microvasculature and changes in metabolic and signalling pathways could contribute to the effects seen after radiation (Monje and Palmer, 2003; Silasi and others, 2004). Our paradigm only suppressed the generation of new neurons in response to seizures. Immature neurons, which had arisen from progenitor division during ongoing neurogenesis in the weeks prior to the radiation, were still available for integration in aberrant circuitry.

Here, we specifically elaborated on the effect of early radiation on kindling epileptogenesis. AD threshold, AD duration and behavioural response were compared between radiated and non-radiated rats during kindling acquisition and retest stimulation, one and two weeks after the end of kindling acquisition in the fully kindled state. Neurogenesis or neuroblast formation was quantified over the same period.

METHODS

ANIMALS

Female Sprague-Dawley rats (200-225 g body weight; Harlan, the Netherlands) were treated according to guidelines approved by the European Ethics Committee (decree 86/609/EEC). The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University Hospital (ECP 06/13). The animals were kept under environmentally controlled conditions (12h normal light/dark cycles, 20-23°C and 50% relative humidity) with food and water *ad libitum*.

SURGERY

All rats were anesthetized with a ketamine (80 mg/kg) and xylazine (7.5mg/kg) mixture (pH 4.0) intraperitoneally and positioned in a stereotactic frame (Bilaney Consultants, Düsseldorf, Germany). After exposure of the skull, 8 small burr holes were drilled for the placement of anchor screws and electrodes. Epidural electrodes were custom made by soldering an insulated copper wire to a screw. Two epidural electrodes were used for the recording of surface EEG. One was placed on the left side of the skull at the height of the frontal cortex and one on the left side at the height of the parietal cortex. One epidural electrode was placed at the height of the sutura lambdoidea and was used as reference/ground electrode. A quadripolar electrode was custom-made by gluing together two recording electrodes (polyimide coated stainless steel wires, 70µm diameter, California Fine Wire, USA) and two stimulation electrodes (polyimide-coated stainless steel wire, 125µm diameter, Plastics One, USA). Each of the wires was cut at a different length so that the electrode tips were separated by 400 µm from each other. This quadripolar registration/stimulation electrode was inserted in the right hippocampus (AP -3.9, ML -1.7, DV -4.0 mm relative to bregma). All four electrode tips were in the hippocampus (fig. 1A). In the remaining four burr holes, anchor screws were placed to fix the electrodes on the skull by dental cement. All electrodes were lead to a connector which could be attached to the EEG cables.

EXPERIMENTAL SETUP

The setup of the experiment is given in fig. 1B and explained below. We repeated the same experiment twice, using 16 rats per session. We pooled the kindling data of both experimental sessions for analysis.

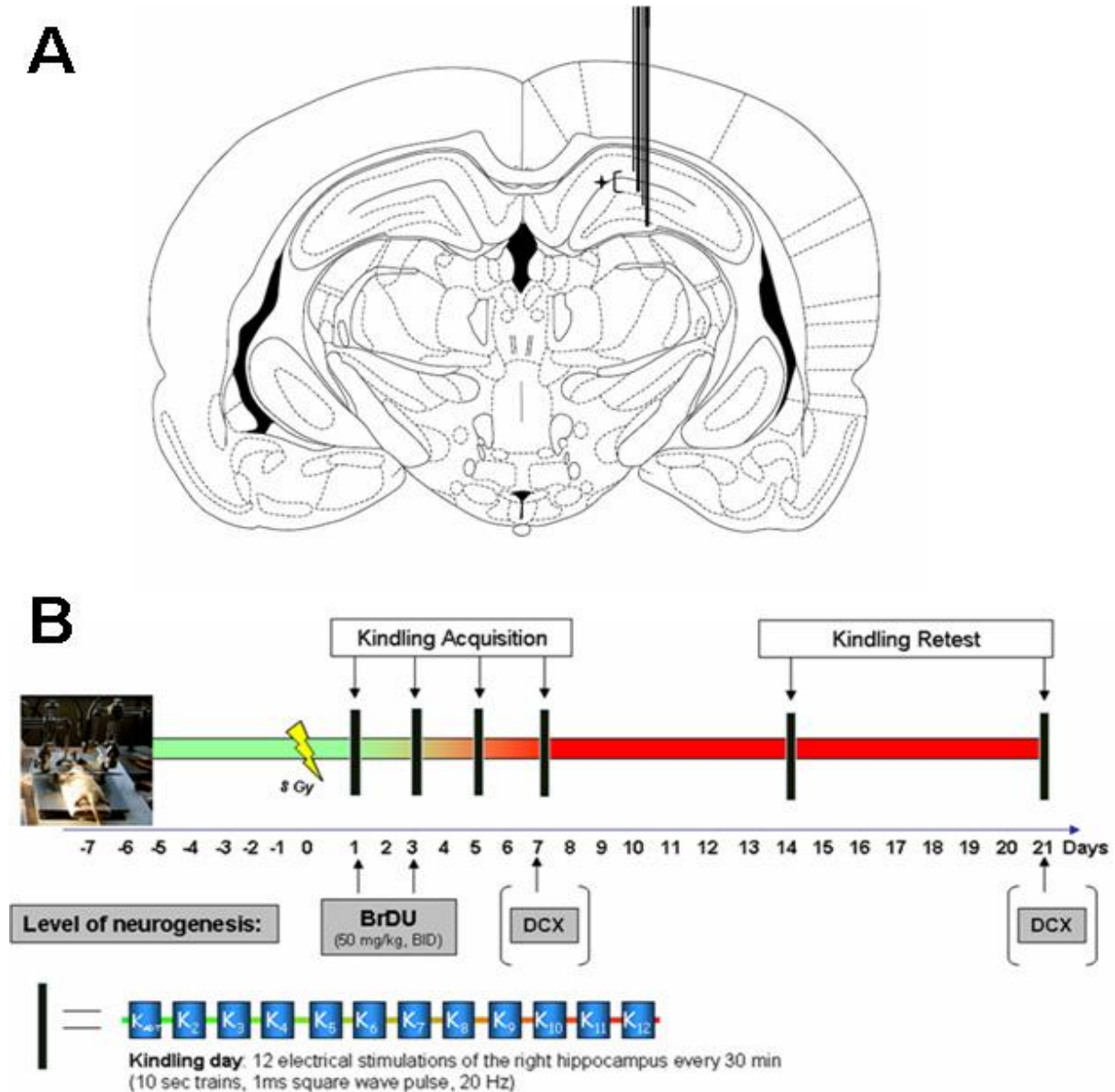


Fig 1: Implantation of registration/stimulation electrode and experimental setup. A: A quadripolar registration/stimulation electrode is stereotactically positioned in the right hippocampus (AP-3.9, ML-1.7, DV-4 mm relative to bregma). All four tips are located in the hippocampus. Hippocampal EEG is recorded on the first and third most dorsal tips. Kindling stimulations are delivered through the second most dorsal and the most ventral electrode tips. Electrode tips are spaced by 0.5 mm \pm . B: Seven days after electrode implantation half of the rats ($n=16$) were exposed to low-dose (8 Gy), whole brain irradiation. On the first and the third day after radiation, rats were injected intraperitoneally with BrdU (50 mg/kg) twice a day to label all dividing cells. Staining hippocampal sections for BrdU at the end of the experiment allowed us to assess the level of neurogenesis in these first days after radiation. One day after radiation all rats (non-irradiated and irradiated) were subjected to a rapid kindling protocol. This protocol consists of an acquisition phase during which rats are stimulated twelve times a day, every 30 minutes, for four alternating days. One and two weeks after termination of the acquisition phase, rats were retested for their kindling response in the kindling retest phase. The number of doublecortin (DCX) positive cells at the end of the experiment indicates the level of neurogenesis at that time point.

AFTERDISCHARGE THRESHOLD DETERMINATION

Following surgery, rats were allowed to recover for at least one week. At the day of radiation, rats were placed in the kindling setup and connected through a commutator to the digital EEG-recording system and to the constant current stimulator. In all rats the minimal stimulation intensity to evoke an afterdischarge in the hippocampus (afterdischarge threshold, ADT) was determined to assure that all rats that would be subjected to the radiation were responsive to kindling stimulation. Therefore a 10 second pulse train (20 Hz, 1 ms square wave pulses) was delivered to the two stimulation contacts of the quadripolar registration/stimulation electrode, placed in the hippocampus. The first stimulation train had a minimal current intensity of 25 μ A. Stimulation intensity was increased with 25 μ A steps until the AD threshold (ADT) was reached and a hippocampal seizure could be detected on the EEG in response to the stimulation.

RADIATION

After determining ADT, 16 rats were randomly divided into two groups: a radiated group (n= 8) in which rats were subjected to low-dose, whole brain radiation and a non-radiated group (n=8) in which rats underwent the same experimental procedures but were not radiated. Before radiation the rats were anesthetized by intraperitoneal injection of a ketamine/xylazine mixture (80mg/kg and 7.5mg/kg respectively, pH 4.0) and fixed in a stereotactic frame (Bilaney Consultants). The radiation was performed with 5 MV photons on a SL75-5 Elekta linear accelerator, at a dose rate of 3.5 Gy per minute. By exposing the rats for 2 minutes and 17 seconds, a total dose of 8 Gy was delivered. A single field of 2 x 2 cm at a distance of 100 cm to the dental cement, fixing the electrodes to the head, was used. The thickness of the dental cement was about 1 cm, compensating for the build-up effect under the skin. The field was centred on the animal's head and its cranial border was put just behind the eyes. The dose at skin level under the dental cement was verified with thermoluminescent dosimetry.

ALTERNATE DAY RAPID KINDLING

In this experiment we used the alternate day rapid kindling protocol described by Lothman and others (Lothman and others, 1985; Lothman and Williamson, 1994). This consists of a kindling acquisition period during which 48 kindling stimulations are given spread over four alternating days, interleaved by days without stimulation. So, rats receive twelve stimuli per day which are separated by 30 minute intervals. The first stimulus for each animal on each day is used to determine the ADT as described above. The remaining 11 kindling stimulations are given at 400 μ A in amplitude, which was always above threshold. The typical behaviour associated with the hippocampal AD's is classified according to the adjusted version of the scale of Racine: stage 1, immobility, eye closure, twitching of vibrissae, facial clonus, wet dog shakes; stage 2: head nodding, chewing, severe facial clonus, wet dog shakes; stage 3: clonus of one forelimb; stage 4: rearing, bilateral forelimb clonus; stage 5: rearing, bilateral forelimb clonus, loss of balance and falling (Racine, 1972). The duration of all afterdischarges was also measured on the EEG. To check for a retained kindling response,

rats were retested one and two weeks after the end of the acquisition phase. On these retest days ADT, seizure stage and ADD were evaluated.

5'-BROMO-2'-DEOXYURIDINE (BRDU) INJECTION

To evaluate neurogenesis in the early phase of kindling all rats received four intraperitoneal 5'-bromo-2'-deoxyuridine (BrdU, Roche diagnostics, Vilvoorde, Belgium) injections (50 mg/kg). Two injections were given on the first day after radiation and two were given at the third day after radiation. The first of two daily BrdU injections was given immediately before the determination of the ADT; the second injection was given immediately after the last (twelfth) kindling stimulation. So, BrdU injections were separated by 6 hours.

TISSUE PREPARATION

Rats were terminally anesthetized using pentobarbital (Nembutal, Sanofi Santé B.V., France; 180 mg/kg) and perfusion-fixed through the ascending aorta with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) and 0.2% glutaraldehyde (pH 7.4). After in situ postfixation overnight (at +4°C), the brains were dissected and transferred to 30% sucrose in PB. After three days at +4°C, the brains were frozen at -20°C to -30°C in 2-methylbutane and stored at -80°C. Coronal and horizontal sections (40µm) were obtained by the use of a freezing microtome and stored in a cryoprotectant (25% ethylene glycol and 25% glycerin in a 0.05 M PB) at -20°C before immunohistochemistry.

IMMUNOHISTOCHEMISTRY

Free floating sections were treated with 0.6% H₂O₂ in Tris-buffered saline (TBS; 0.15 M NaCl and 0.1 M Tris-HCl, pH 7.5) for 30 min to block endogenous peroxidase activity. For immunohistochemical detection of incorporated BrdU, DNA denaturation was performed in the following manner: tissue was incubated in 50% formamide and 2× SSC (0.3 M NaCl and 0.03 M sodium citrate) for 2 hr at 65°C, rinsed for 15 min in 2× SSC, incubated again for 30 min in 2 M HCl at 37°C, and rinsed for 10 min in 0.1 M boric acid at pH 8.5. To stain for doublecortin (DCX) and vimentin, sections were pre-treated for 30 min with preheated (80°C) NaCitrate (10mM, pH9) before blocking of endogenous peroxidase activity.

Following extensive washes in TBS, sections were blocked with a solution containing TBS, 0.25% Triton X-100 and 3% donkey serum for 30 min. The same solution was used during the incubation with primary antibodies. Primary antibodies were applied overnight at 4°C. For chromogenic immunodetection, tissue sections were rinsed three times and incubated for 2 hr with biotinylated donkey secondary antibodies. After extensive washes in TBS an avidin-biotin-peroxidase complex solution (Vectastain Elite ABC-kit Standard, Vector Laboratories, Burlingame, CA) was applied for 1 hr before 5 min of peroxidase detection (using 0.25 mg/ml 3,3'-diaminobenzidine, 0.01% H₂O₂, and 0.04% NiCl). Sections were placed on Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany), alcohol dehydrated and mounted in Neo-mount (Merck, Darmstadt, Germany).

For epifluorescence detection sections were washed extensively and incubated with fluorochrome-conjugated species-specific secondary antibodies. Sections were placed on Superfrost Plus slides and mounted in vectashield mounting medium (H-1000, Vector Laboratories).

The following antibodies and final dilutions were used. Primary antibodies: mouse anti-BrdU (1:400; Roche Diagnostics, Vilvoorde, Belgium), rat anti-BrdU (1:250, Nordic Biosite AB, Täby, Sweden), goat anti-DCX (1: 125, Santa Cruz Biotechnology, Heidelberg, Germany), mouse anti-vimentin (1:25, DAKO, Heverlee, Belgium), mouse anti CD11b/c (OX42, 1:100, BD Biosciences, Erembodegem, Belgium), mouse anti-NeuN (1:100, Chemicon, Temecula, CA, USA). Secondary antibodies: biotinylated donkey anti-mouse IgG (1:1000, JacksonImmunoResearch, Suffolk, England), biotinylated donkey anti-goat IgG (1:1000, JacksonImmunoResearch), donkey anti-rat-Alexa488 (1:1000, Molecular Probes, Eugene, Oregon, USA) and donkey anti-mouse-Alexa555 (1:1000, Molecular Probes).

QUANTIFICATION

The number of BrdU and DCX-positive cells was determined in the granule cell layer (GCL) [including the subgranular zone (SGZ)] in six (BrdU) or twelve (DCX) immunoperoxidase-stained, 40- μ m-thick coronal sections taken 480 μ m (BrdU) or 240 μ m (DCX) apart. The numbers of positive cells were counted within the GCL and two cell diameters below the GCL (representing SGZ), ignoring the cells in the uppermost focal plane and focusing through the thickness of the section to avoid errors caused by oversampling (Gundersen and others, 1988; Coggeshall and Lekan, 1996). Sample and GCL area estimations were obtained by planimetry, using a Lucivid device (MicroBrightField, Colchester, VT) attached to a Leica microscope. The section thickness of 40 μ m was used in the estimation of dissector sample volume. Section thickness and intersection distance were used for the estimation of absolute GCL volume. Cell numbers were expressed as cells per GCL.

Using a laser-scanning confocal microscope (Leica, TCS-NT, Leica Microsystems, Bensheim, Germany) we determined the percentage of newly generated cells differentiating into neurons. Therefore BrdU positive cells within the GCL and SGZ were randomly selected and analysed for BrdU / NeuN double labelling. In total we evaluated 612 BrdU labelled cells in the non-radiated group and 207 BrdU labelled cells in the radiated group.

To assess for inflammation, numbers of vimentin and CD11b/c positive cells were counted in four high power fields (200x), selected from four defined hippocampal regions (CA1, CA3, hilus and granule cell layer) per section. This was done on four mid-dorsal sections, spaced by 480 μ m, of the kindled hippocampus.

DATA ANALYSIS

All data (kindling parameters and cell counts) were measured and scored in a blind manner. Before analysis, normality of the data was assessed using the Kolmogorov-Smirnov test. A parametric T-test was used to compare normally distributed data; a non-parametric Mann-Whitney U test was used to compare data which

was not normally distributed. All data were expressed as means and standard error of the mean. $P < 0.05$ was assumed to indicate a significant difference.

ADT's were compared using the Mann-Whitney U test. For every kindling day, the mean seizure stage and mean ADD was calculated for each rat. Seizure stages were compared between groups using the Mann-Whitney U-test, ADD's were compared using the Student's *t* test.

To assess progression of seizure severity within one kindling day, seizure stages of each individual rat were plotted as a function of stimulation number. Since the time between two stimulations in all rats was exactly 30 minutes, stimulation number could be multiplied by 30 minutes and time values could be used as abscissa of the seizure progression curves. Using a curve-fitting program Kaleidograph (Synergy Software) an exponential function with stage 5 as a maximal attainable value could be fitted to the seizure progression curve of each rat. Time constants of these functions were determined and compared between groups using a T-test to check for differences in seizure stage progression rate.

Numbers of BrdU labelled cells, CD11b/c positive microglia and vimentin positive astrocytes were compared between groups using the Mann Whitney U-test. Number of DCX labelled neuroblasts were compared using the T-test.

RESULTS

EFFECT OF RADIATION ON NEUROGENESIS AND NEUROBLAST FORMATION

To assess for radiation-induced differences in the rate of neurogenesis during the early kindling acquisition phase, BrdU was injected twice a day on the first and second kindling acquisition session, respectively one and three days after radiation (fig. 1B). Three weeks after radiation the rats were killed. In four, randomly chosen, radiated and non-radiated rats the number of BrdU-labelled cells in the GCL (including SGZ) were counted and the fraction of BrdU positive cells which double-labelled for NeuN was determined. In radiated rats we found 545 ± 157 BrdU-positive cells per GCL which was significantly lower ($p < 0.01$, Mann-Whitney U test) and only 17% of the level in control, non-radiated rats (3134 ± 998 cells/GCL, fig 2A). By analyzing the fraction of BrdU positive cells that double-labelled for NeuN, we found no difference between radiated and non-radiated rats. In radiated rats $81 \pm 6\%$ and in non-radiated rats $79 \pm 2\%$ of the BrdU positive cells were labelled for NeuN. This indicates that the fraction of newborn cells that differentiate towards neurons during the first three days of kindling acquisition and that survive for at least three weeks was not affected by the radiation. Taken together, based on the BrdU positive cells counts, we found that neurogenesis in radiated rats during the first three days after radiation was reduced to about 17 % of the level in non-radiated rats. Representative examples of BrdU staining in radiated and non-radiated rats and double-labelling for BrdU and NeuN are given in fig 2B.

In the same rats, used for quantification of BrdU positive cells, the amount of DCX positive neuroblasts in the SGZ and GCL was determined three weeks after radiation and after 70 kindling stimulations (fig 3). The number of DCX labelled cells 21 days after radiation was 11378 ± 1399 cells/GCL in radiated rats which was 44 % of the number in non-radiated rats (25741 ± 2134 cells/GCL, $p < 0.01$, T-test, fig 3A). In order to investigate whether neuroblast formation was more efficiently suppressed in the first week after radiation, during kindling acquisition, we repeated the experiment. In this session half of the rats (four radiated and four non-radiated) were killed one week after radiation, at the end of the kindling acquisition, to quantify the number of DCX-positive neuroblasts at that time point. In radiated rats 863 ± 203 DCX expressing neuroblasts / GCL were found. This was only 5% of the 17782 ± 2134 neuroblasts/GCL that were counted in non-radiated rats ($p < 0.01$, T-test, fig 3A). These results show an almost complete suppression of neuroblast production at the end of the acquisition phase. Fig 3A illustrates the partial rebound of neuroblast production in radiated rats from 5% (7 days post radiation) to 44% (21 days post radiation) of non-radiated, control levels.

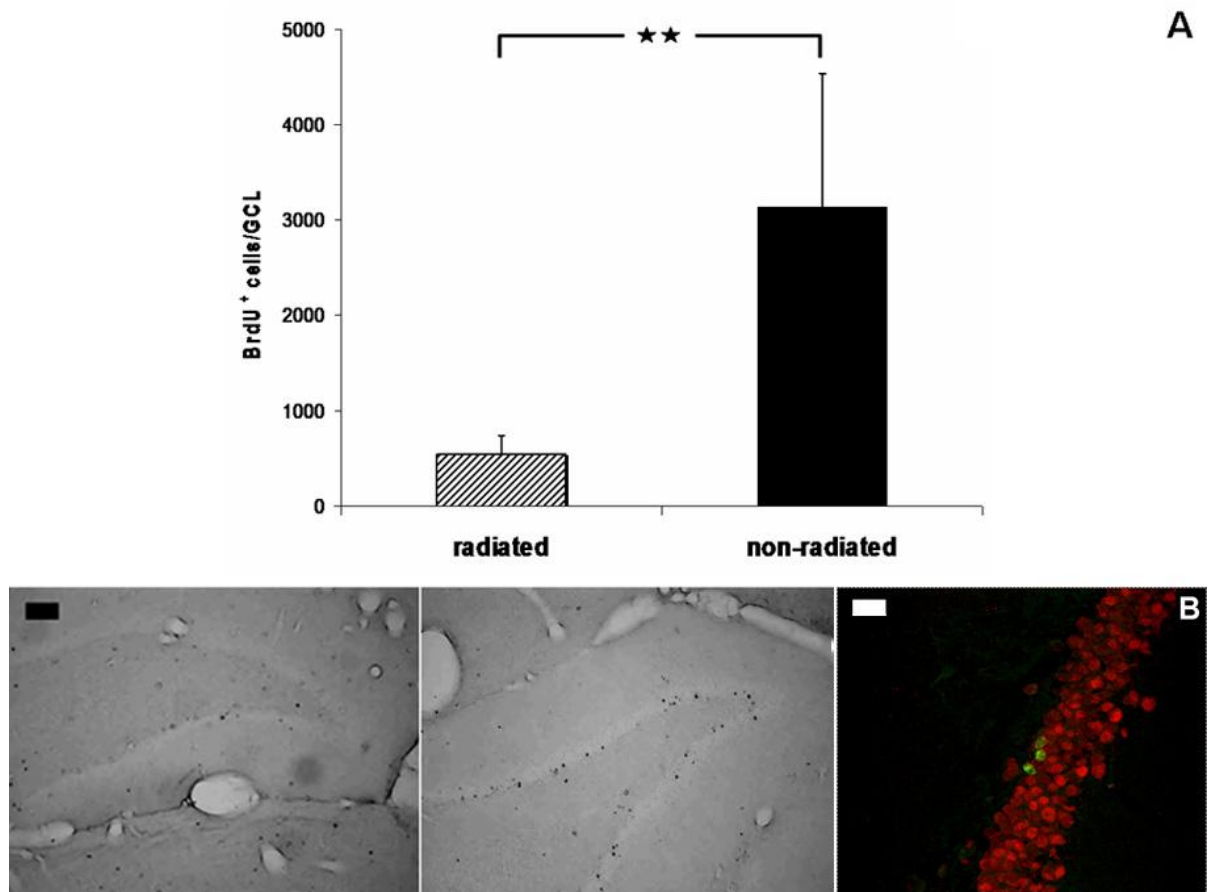


Fig 2: Level of neurogenesis during the first days of radiation: BrdU was injected in rats twice daily on day 1 and day 3 after radiation. Twenty-one days after radiation, after 70 kindling stimulations, rats are sacrificed and the number of BrdU positive cells in the SGZ and the GCL, in four radiated and four non-radiated rats, were counted in six coronal sections taken 480 μ m apart. The volumes of the GCL were determined and the results were expressed as number of cells per GCL. A: In radiated rats 545 \pm 157 BrdU labelled cells/GCL were found while in non-radiated rats 3134 \pm 998 cells/GCL were counted. As the fraction of BrdU labelled cells co-expressing NeuN was highly comparable (81% in radiated rats compared to 79% in non-radiated rats), the difference in BrdU positive cell counts reflects a reduction in neurogenesis in radiated rats to levels which are 17% of the neurogenesis in non-radiated rats. $\star\star = p < 0.01$. B: Representative examples of chromogenic staining for BrdU in radiated (left) and non-radiated rats (middle) (scale bar = 200 μ m). The right picture is an example of fluorescent doublestaining for BrdU (green) and NeuN (red) (scale bar= 20 μ m)

For both the radiated and non-radiated groups, the number of DCX positive cell numbers was significantly lower at the end of the acquisition phase (after 48 kindling stimulations) than at the end of the retest phase (after 70 kindling stimulations), which could be explained based on the stimulatory effect of seizures on proliferation. However, one needs to be aware that numbers of DCX positive neuroblasts counted on day 7 and day 21 after radiation involves two separate experiments which makes it difficult to compare numbers. Representative examples of DCX staining in radiated and non-radiated rats at the end of kindling acquisition are illustrated in respectively fig 3B and 3C. DCX stainings in rats three weeks after radiation are shown in fig 3D (radiated) and 3E (non-radiated).

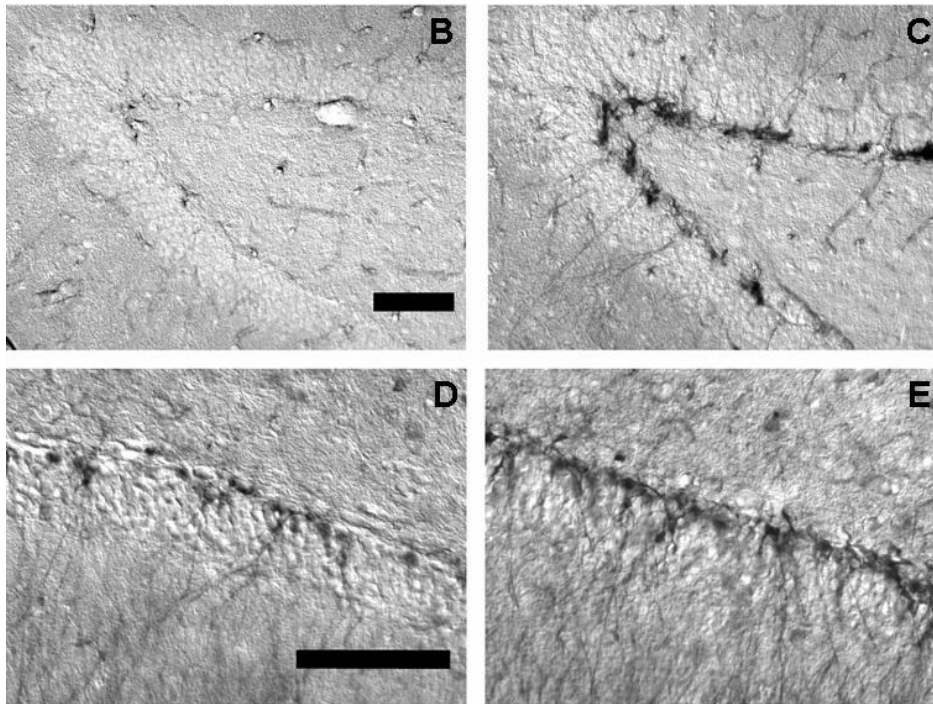
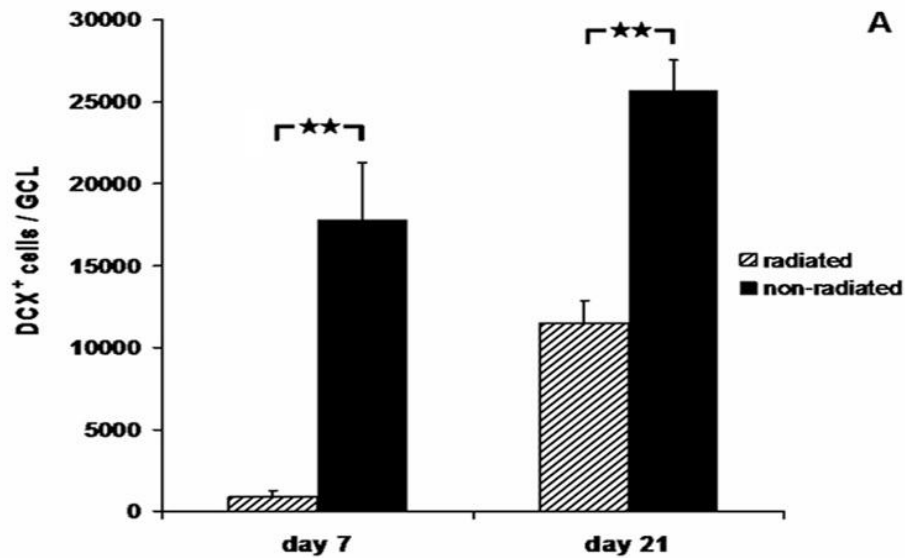


Fig 3: Level of neurogenesis at the end of the kindling acquisition and retest phase: To assess for differences in neuroblast production at the end of the kindling acquisition phase (one week after radiation) and at the end of the kindling retest phase (three weeks after radiation) the number of DCX positive cells in the SGZ and the GCL was counted in four radiated and non-radiated rats. Cells were counted in twelve immunoperoxidase-stained, 40-μm-thick coronal sections taken 240 μm apart. The volumes of the GCL were determined and the results were expressed as number of cells per GCL. A: Seven days after radiation and after 48 kindling stimulations 863 ± 203 DCX⁺ neuroblasts / GCL were found in radiated rats and 17782 ± 2134 neuroblasts / GCL in non-radiated rats. This shows that the level of neurogenesis in radiated rats is only 5 % of the level in non-radiated controls. Three weeks after radiation and after 70 kindling stimulations 11378 ± 1399 cells/GCL were found in radiated and 25741 ± 2134 cells/GCL in non-radiated rats. This shows that the level of neurogenesis in radiated rats is 44 % of the level in non-radiated controls. ** = $p < 0.01$. B: DCX staining in the hippocampus of radiated rats at the end of the kindling acquisition, seven days after radiation. C: DCX staining in the hippocampus of non-radiated rats at the end of the kindling acquisition, seven days after radiation. D: DCX staining in the hippocampus of radiated rats 21 days after radiation. E: DCX staining in the hippocampus of non-radiated rats 21 days after radiation. Scale bar = 100 μm.

EFFECT OF RADIATION ON AD THRESHOLD

In all rats, used in this study, the stimulation threshold to evoke an afterdischarge (fig. 4A) was measured at the beginning of each kindling day. The ADT remained below 200 μ A in all rats throughout the experiment. During the first two kindling days ADT did not significantly differ between radiated and non-radiated rats (fig. 4B). At kindling day 1 ADT was respectively 50 ± 3 μ A and 56 ± 3 μ A. At kindling day 2 levels were respectively 57 ± 3 μ A and 72 ± 6 μ A. However, as kindling acquisition progressed, ADT increased further in non-radiated rats while it remained around 60 μ A in radiated rats. This resulted in significantly lower ADT levels in radiated rats compared to non-radiated rats. The difference reached significance ($p < 0.05$, Mann-Whitney U test) on kindling day 3: 64 ± 4 μ A in radiated rats versus 83 ± 7 μ A in non-radiated rats. This difference remained significant ($p < 0.01$, Mann-Whitney U test) on kindling day 4 (66 ± 3 μ A versus 92 ± 7 μ A) and was still observed at retest day 1 (59 ± 4 μ A versus 91 ± 14 μ A, $p < 0.01$, Mann-Whitney U test) and retest day 2 (60 ± 4 μ A versus 89 ± 8 μ A, $p < 0.01$, Mann-Whitney U test).

EFFECT OF RADIATION ON AD DURATION

The AD duration was longer on acquisition day 2 than on acquisition day 1 in all rats irrespective of treatment. For the remaining kindling days of the acquisition and retest phase the AD duration remained stable in all rats. No difference in AD duration could be demonstrated between radiated and non-radiated rats neither during kindling acquisition phase nor during retest phase (fig. 4C).

EFFECT OF RADIATION ON BEHAVIOURAL STAGE

At acquisition day 1 and 2 no difference in mean seizure severity could be detected between radiated and non-radiated rats (fig. 4D). However, at acquisition day 3 and 4 mean seizure severity was significantly higher in radiated compared to non-radiated rats. At kindling day 3 mean seizure stage in radiated and non-radiated rats was respectively 4.0 ± 0.1 and 3.4 ± 0.1 ($p < 0.05$; Mann-Whitney U test). At kindling day 4, radiated rats still displayed more severe seizures than non-radiated rats with the mean seizure stage being 3.8 ± 0.1 and 3.2 ± 0.1 respectively ($p < 0.01$; Mann-Whitney U test).

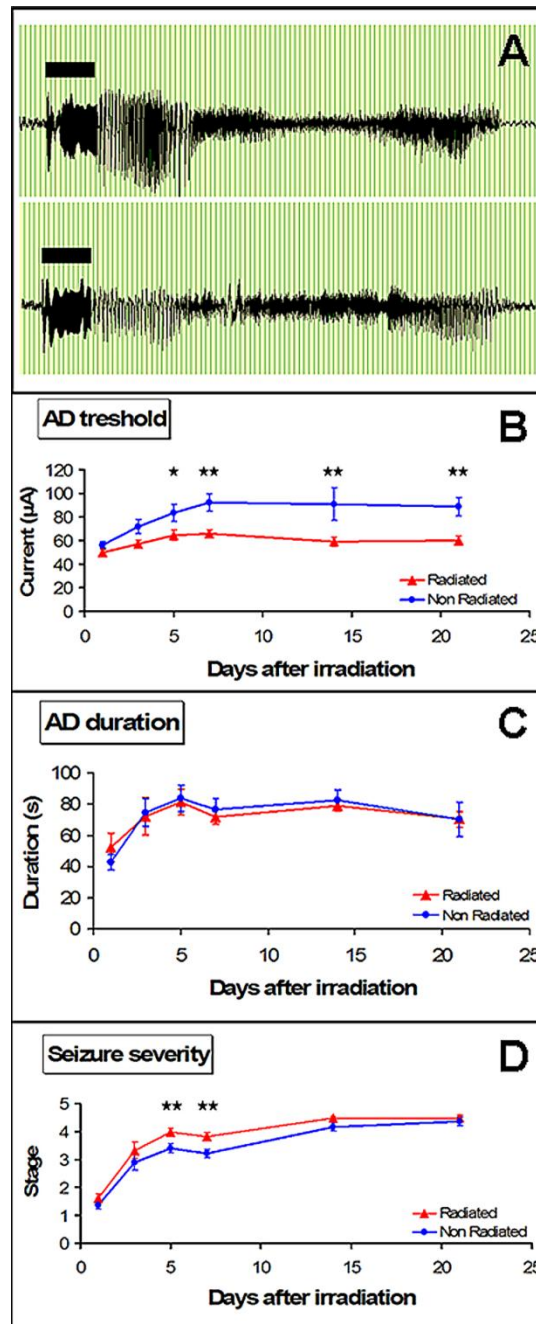


Fig 4: Rapid kindling parameters: The effect of radiation on kindling was evaluated by three outcome parameters: the afterdischarge threshold, the afterdischarge duration and seizure severity stage. A: Representative example of a hippocampal afterdischarge in non-radiated (top) and radiated (bottom) rats. The bar above each EEG trace represents 10 seconds which is also the duration of the kindling stimulation. B: Evolution of the afterdischarge threshold (ADT), measured at the start of each kindling day. From the graph it is evident that during the first two kindling days ADT did not significantly differ between radiated and non-radiated rats. During the remaining of the kindling, ADT remained significantly lower in radiated compared to non-radiated rats with stimulation intensity being respectively $64.3 \pm 4.3 \mu\text{A}$ and $83.3 \pm 7.2 \mu\text{A}$ at kindling day 3; $66.1 \pm 3.3 \mu\text{A}$ and $92.2 \pm 7.1 \mu\text{A}$ at kindling day 4; 59.1 ± 3.8 and $90.9 \pm 14 \mu\text{A}$ at retest day 1 and $60.0 \pm 4.1 \mu\text{A}$ compared to $88.6 \pm 7.8 \mu\text{A}$ at retest day 2. C: The mean duration of all twelve afterdischarges, determined for each kindling day, did not differ between radiated and non-radiated rats, throughout kindling acquisition and retest phase. D: Mean seizure severity stage on kindling day 3 and 4 is significantly higher in radiated compared to non-radiated. At kindling day 3 mean seizure stage of radiated rats is 4.0 ± 0.1 compared to 3.4 ± 0.1 in non-radiated rats. At kindling day 4 mean seizure stages are respectively 3.8 ± 0.1 compared to 3.2 ± 0.1 . This difference is no longer evident on both kindling retest days. Scale bar = 200 μm ; * = $p < 0.05$; ** = $p < 0.01$

Retesting the rats one and two weeks after the fourth kindling day showed that this difference in mean seizure severity was not longer evident. Apparently the difference in treatment did not prevent the rats from reaching the fully kindled state.

At retest day 1 mean seizure stage in radiated was 4.5 ± 0.1 and 4.2 ± 0.1 in non-radiated rats. At retest day 2 mean seizure stage was respectively 4.5 ± 0.1 and 4.4 ± 0.2 (n.s., Mann Whitney U test).

For each rat, seizure stage acquisition within one kindling day could also be illustrated by plotting seizure stage as a function of time. The time points as abscises of the plots were obtained by multiplying stimulation number by 30 minutes which is the fixed time period between two stimulations. For each rat this resulted in a “severity progression curve” to which an exponential function could be fitted with stage 5 as the maximal attainable value. The time constant of this exponential curve reflected the rate by which seizure severity increased during the day. Time constants were significantly lower in radiated compared to non-radiated rats on kindling day 3 ($p < 0.01$, T-test) and kindling day 4 ($p < 0.05$, T-test). On kindling day 3 mean time constants were 90 ± 14 min for radiated and 151 ± 16 min for non-radiated rats. On kindling day 4 mean time constants were respectively 108 ± 12 min and 168 ± 18 min. On both retest days, time constants were no longer different between radiated and non-radiated rats. The mean time constants were respectively 39 ± 8 minutes versus 61 ± 11 min on retest day 1 and 38 ± 9 min versus 50 ± 14 min on retest day 2. So, on kindling day 3 and 4 radiated rats progressed more rapidly to the most severe seizure than non-radiated rats but this was no longer evident on both retest days. This is also illustrated in fig. 5 which shows the mean seizure severity curves of both groups and the mean exponential functions which fit best to the progression curves, on acquisition day 3 (fig. 5A) and 4 (fig. 5B) and on both retest days (fig. 5C and 5D).

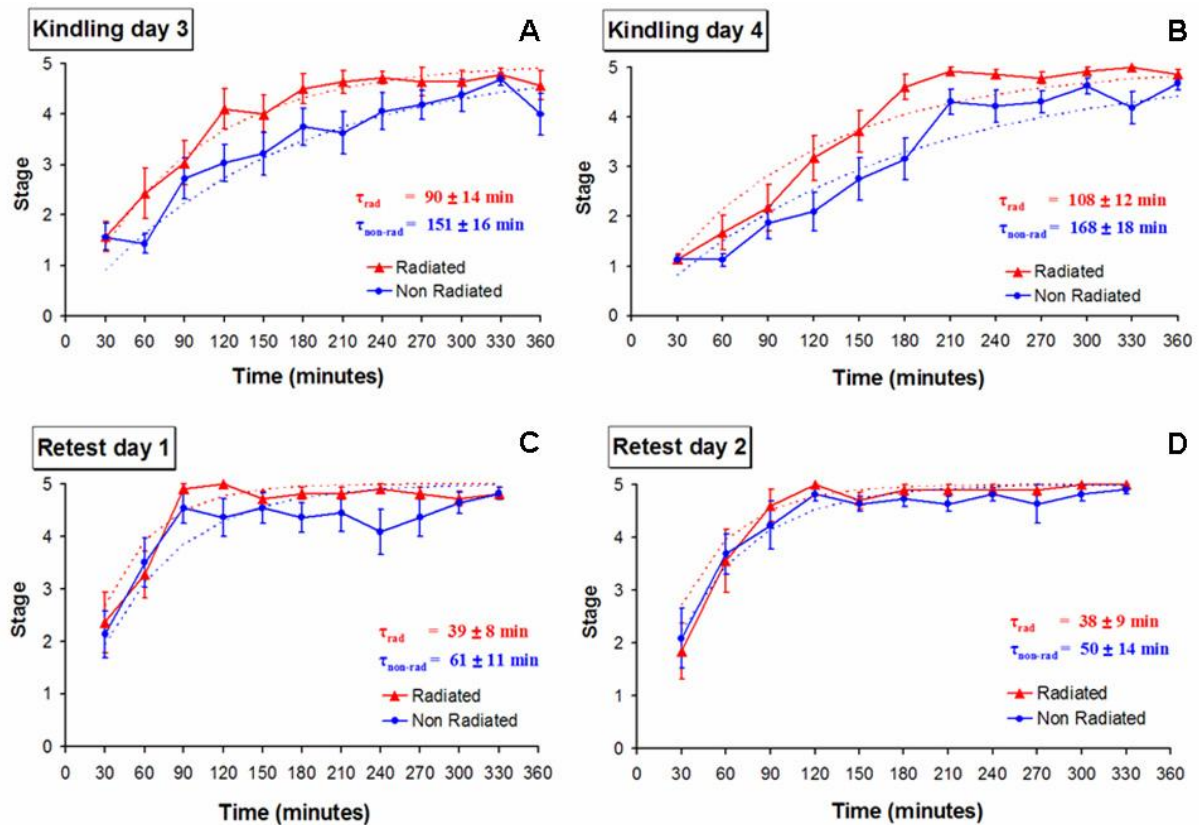


Fig 5: Seizure stage progression on individual kindling days: To evaluate seizure stage progression during one day, seizure stages could be plotted as a function of time. Time points in the X-axis were calculated by multiplying stimulation number with 30 minutes, the exact time between two stimulations. To this “severity progression curve” an exponential function can be fitted which is characterized by a maximum amplitude of stage 5 and a time constant, τ . Time constants were significantly lower in radiated compared to non-radiated rats on kindling day 3 ($p < 0.01$, T-test) and 4 ($p < 0.05$, T-test). This difference was no longer evident on both retest days. This figure shows mean seizure progression curves (—) and the mean exponential function which fits best to the progression curves (----) on kindling day 3 (A) and kindling day 4 (B) and both retest day (C, D).

EFFECT OF RADIATION ON INFLAMMATION

We found CD11b/c positive reactive microglia and vimentin positive reactive astrocytes both in kindled, non-radiated (fig 6A-D) and kindled, radiated rats (fig 6E-6H) at the end of the acquisition phase, seven days after radiation. Quantitative analysis could not reveal differences in the number of activated microglia between non-radiated (100 ± 15 cells/high power field; fig 6 A, 6B) and radiated rats (78 ± 17 cells/high power field; fig 6E, 6F). We also could not detect differences in the number of reactive astrocytes in non-radiated (106 ± 12 cells/high power field; fig 6C,6D) compared to radiated rats (101 ± 4 cells/high power field; fig 6G,6H). This indicates that radiation has no or only little effects on kindling induced inflammation at day seven after radiation through activated microglia or astrocytes.

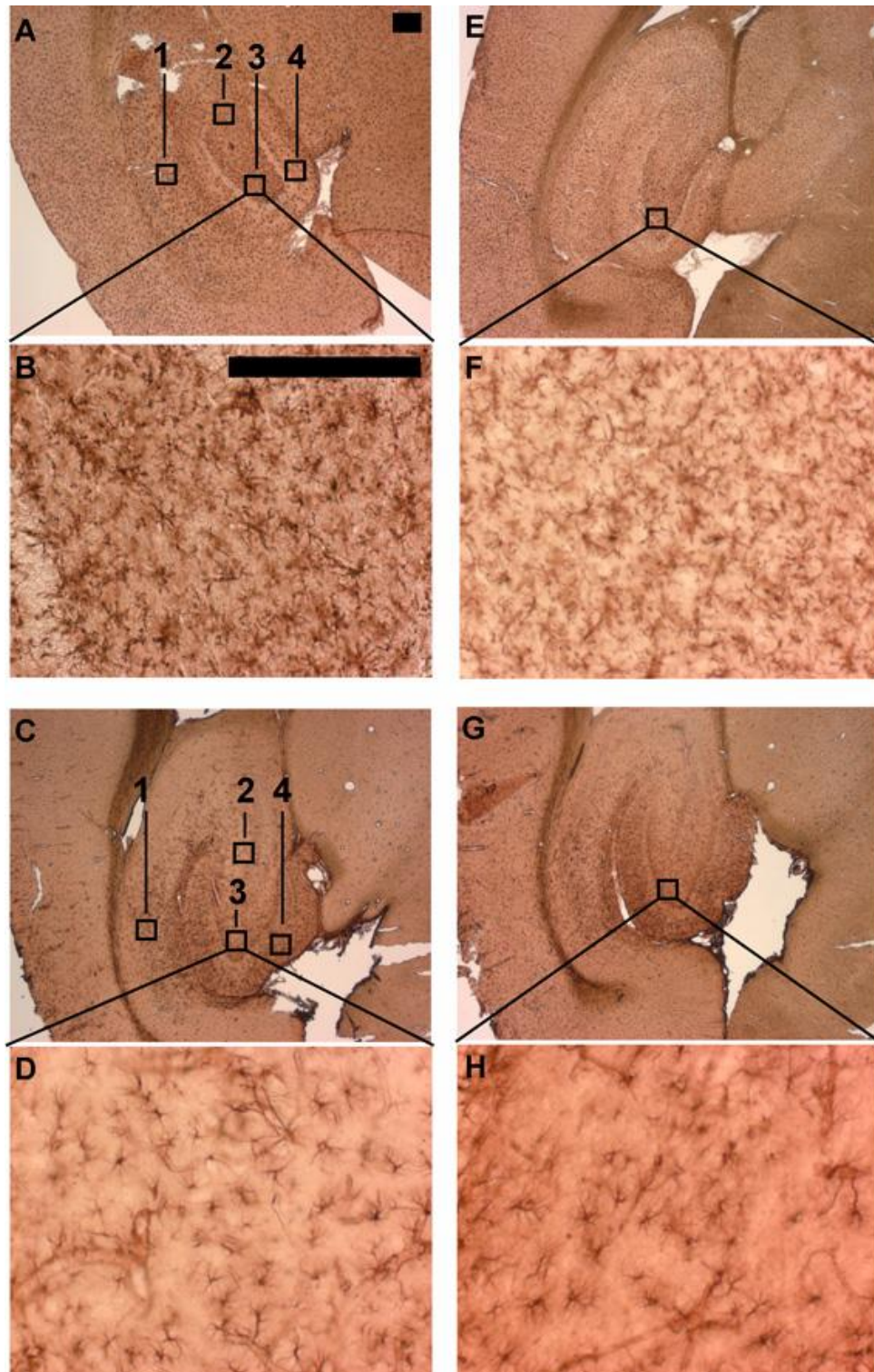


Fig 6: Inflammation at the end of kindling acquisition: Both in non-radiated (A-D) and radiated (E-H) kindled rats CD11b/c positive activated microglia (A, B, E, F) and vimentin positive reactive astrocytes (C, D, G, H) were present in different brain regions. To compare between groups CD11b/c (A) and vimentin (C) positive cells were counted in four high power fields selected from distinct regions of the stimulated hippocampus (1: CA1 region, 2: CA3 region, 3: hilus and 4: granule cell and molecular layer of dentate gyrus). B and F show a representative example of a high power image selected from the hilar region containing CD11b/c positive microglia. D and H show a representative example of a high power image selected from the hilar region containing vimentin positive reactive astrocytes. From the images and the quantitative analysis no significant difference in number of reactive microglia and astrocytes were evident. Scale bar = 200µm

DISCUSSION

This study shows that the exposure of rats to whole brain low dose (8Gy) γ -radiation one day before the initiation of rapid hippocampal kindling significantly reduces seizure-induced neurogenesis and neuroblast formation during kindling acquisition. Radiating rats just prior to kindling does not prevent epileptogenesis but significantly affects several parameters of the kindling acquisition. These kindling parameters are modified in a way which reflects higher excitability in response to stimulation. Firstly, rats that have been radiated show less increase in the threshold to evoke an afterdischarge compared to non-radiated controls. This leads to a significantly lower ADT in radiated rats which becomes evident five days after radiation and remains significantly lower for at least three weeks after radiation. This represents a higher excitability of hippocampal networks. Secondly, although radiation has no effect on the duration of afterdischarges radiated rats acquire more rapidly more severe seizures at the end of the kindling acquisition phase (five to seven days after radiation) compared to non-radiated rats.

Other studies have investigated the effect of radiation on the course of amygdala kindling epileptogenesis (Jenrow and others, 2001; Jenrow and others, 2006). A high radiation dose of 25 Gy, applied focally to the amygdala prior to kindling, does not affect stimulation threshold to evoke a generalized seizure but causes an increased tendency for seizure activity to propagate into brain stem circuits. However if radiation is applied later, when the kindled state is already established, the tendency of seizures to spread into brain stem circuits is decreased (Jenrow and others, 2001; Jenrow and others, 2006). Next to the fact that the radiation is applied much more focal in these studies, the authors also use a radiation dose which is much higher than in our study. Both aspects make it difficult to compare the results of these studies with ours.

By keeping the radiation dose as low as possible, we wanted to minimize other side effects of radiation such as changes in dendritic branching, vasculature, demyelination or radionecrosis, which have been reported to appear after radiation at doses of 20 Gy or higher (Calvo and others, 1988; Hodges and others, 1998; Raber and others, 2004; Wojtowicz, 2006). Conflicting reports are found in literature about inflammation induced by low doses of radiation. Some reports describe chronic inflammation as the major side effect of radiation (Monje and others, 2003; Monje and Palmer, 2003). However other studies cannot demonstrate any inflammation after low dose radiation (Snyder and others, 2005; Wojtowicz, 2006). In our study we find inflammatory astrogliosis and microglial response in both kindling groups but we can not demonstrate differences in extent of this response due to radiation. Although our study can not rule out that radiation induces inflammation, it argues against the possibility that a difference in inflammation, though reactive microglia and astrocytes, is responsible for the differences seen in hippocampal excitability and kindling rate one week after radiation.

Our study demonstrates that there is a recovery in neuroblast formation of up to 50% at the end of the experiment. This is in contrast to other radiation studies, which use a single low dose (10 Gy) radiation and see a stable and prolonged suppression of precursor proliferation and neurogenesis after radiation (Tada and others, 2000; Monje and others, 2002; Mizumatsu and others, 2003; Snyder and others, 2005). Whether, next

to a rebound of neuroblasts formation, there is also partial recovery of functional neurogenesis cannot be concluded from this study. A way to do so would be to repeat the experiment but inject BrdU with a delay of some weeks after the radiation and then look for BrdU/NeuN positive cells some weeks after labelling. The partial restoration of neuroblasts production, three weeks after radiation, could be the result of stimulatory effects of seizures on neuroblast proliferation (Jessberger and others, 2005). A similar recovery in the proliferation of hippocampal neuroblasts is reported in rats eight days after 5Gy radiation, which was applied 24 hours before induction of status epilepticus. This recovery is prevented by delivering a second 5Gy radiation dose four days after SE (Parent and others, 1999). In our experiments a suppression of neurogenesis during the week of kindling acquisition is sufficient so we chose to keep the radiation dose as low as possible and not to radiate a second time during the process of kindling to minimize side effects. However, if longer suppression of neurogenesis would be required it seems that delivery of two fractionated radiation doses of 10 Gy spaced by 24 hours is very effective even after pathological stimulation (Snyder and others, 2005; Wang and others, 2005).

It is evident from studies on neurogenesis in different animal models for TLE that status epilepticus as well as single seizures can promote neurogenesis in the dentate gyrus of the hippocampus (Bengzon and others, 1997; Gray and Sundstrom, 1998; Scott and others, 1998; Scharfman and others, 2000; Nakagawa and others, 2000; Scott and others, 2000; Madsen and others, 2000; Mohapel and others, 2004; Smith and others, 2005; Parent and others, 2006). In addition to an increased generation of neuroblasts, seizures also promote the synaptic integration of newborn neurons (Overstreet-Wadiche and others, 2006). Part of the newborn neurons appears to be important in the reorganization of hippocampal networks, typical for TLE. They migrate towards ectopic locations in the hippocampus, contribute to mossy fiber sprouting and receive inputs of sprouted mossy fibers, including on their basal dendrites (Scharfman and others, 2000; Scharfman and others, 2003; Pierce and others, 2005; Shapiro and Ribak, 2005; Parent and others, 2006; Shapiro and Ribak, 2006).

Our results indicate that seizure-induced neurogenesis has no major contributing role in kindling epileptogenesis since blocking the birth of new neurons during kindling acquisition does not slow down kindling epileptogenesis nor prevents the rats from becoming fully kindled. However, as we radiated the animals only one day before kindling, we cannot exclude that immature granule neurons, generated during ongoing neurogenesis in the weeks before kindling, survived the radiation and contributed to the formation of aberrant circuitry by abnormal migration or the formation of aberrant synaptic connections. A recent study demonstrated that next to neurons generated after SE, neurons born two days before SE showed accelerated maturation and received excitatory perforant-path input earlier than newborn granule cells in normal brains. Part of these neurons were also targets for polysynaptic innervation, likely from mossy fiber recurrent collaterals (Overstreet-Wadiche and others, 2006). So it could be that late phases of ongoing neurogenesis, which are not affected by radiation, contribute to kindling epileptogenesis.

In our study, we find that 8Gy radiation induces alterations of kindling parameters which reflect less inhibition in the hippocampus and a higher tendency of rats to display generalized tonic-clonic seizures during kindling

acquisition. During the course of rapid hippocampal kindling we report an increase in ADT in non-radiated rats, but not in radiated rats. This increase in threshold is typically seen when kindling stimulations are delivered in the dentate gyrus of the dorsal hippocampus and was first described by Racine and coworkers (Racine and others, 1977). Since then, other studies have also reported an increase in inhibition in the dentate gyrus during or after hippocampal kindling (Tuff and others, 1983; Maru and Goddard, 1987; Milgram and others, 1995; Nusser and others, 1998; Gutierrez and Heinemann, 2001). A potential mechanism for this increased inhibition is suggested to be the release of GABA by granule cells neurons (Schwarzer and Sperk, 1995; Gutierrez and Heinemann, 2001; Gutierrez, 2002; Gomez-Lira and others, 2002).

Radiation suppresses seizure-induced neurogenesis and leads to an enhanced excitability; however, many factors besides neurogenesis could be involved in this process. We cannot exclude that other side effects of low dose radiation such as formation of free radicals, effects on metabolic and signalling pathways or changes in microvasculature contribute to the differences observed between radiated and non-radiated kindled rats (Monje and others, 2002; Silasi and others, 2004; Wojtowicz, 2006). To make assumptions on possible causative relationships, we need to work out new model systems in which we are able to very selectively block hippocampal neurogenesis in response to seizures without directly affecting other brain regions or functions. One alternative model in which seizure-induced neurogenesis is blocked and the effect on epileptogenesis has been investigated is the continuous infusion of cytosine-b-D-arabinofuranoside (AraC) into the ventricles of rats that have experienced a pilocarpine induced status epilepticus. In this model a significant reduction of the frequency and duration of spontaneous seizures was demonstrated, while no difference in severity score could be demonstrated (Jung and others, 2004). These results are in contrast to the relationship we found in our study but they also do not allow drawing conclusions about a possible causative relationship between decreased neurogenesis and less epilepsy since AraC also blocks gliogenesis, which is enhanced in the pilocarpine model.

Taken together, our study shows that low-dose brain radiation suppressed seizure-induced neurogenesis during kindling acquisition and caused an enhanced excitability of hippocampal networks. It slightly accelerated kindling, but had little effect on the finally reached epileptic state. This suggests that seizure-induced neurogenesis could play a role in epileptogenesis, but on the other hand also that (short duration) block of neurogenesis does not critically affect the final outcome of kindling epileptogenesis.

REFERENCES

- Alvarez-Buylla A, Herrera DG, Wichterle H. 2000. The subventricular zone: source of neuronal precursors for brain repair. *Prog Brain Res* 127:1-11.
- Bengzon J, Kokaia Z, Elmer E, Nanobashvili A, Kokaia M, Lindvall O. 1997. Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. *Proc Natl Acad Sci U S A* 94(19):10432-7.
- Bonde S, Ekdahl CT, Lindvall O. 2006. Long-term neuronal replacement in adult rat hippocampus after status epilepticus despite chronic inflammation. *Eur J Neurosci* 23(4):965-74.
- Calvo W, Hopewell JW, Reinhold HS, Yeung TK. 1988. Time- and dose-related changes in the white matter of the rat brain after single doses of X rays. *Br J Radiol* 61(731):1043-52.
- Coggeshall RE, Lekan HA. 1996. Methods for determining numbers of cells and synapses: a case for more uniform standards of review. *J Comp Neurol* 364(1):6-15.
- Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH. 1998. Neurogenesis in the adult human hippocampus. *Nat Med* 4(11):1313-7.
- Ferland RJ, Gross RA, Applegate CD. 2002. Increased mitotic activity in the dentate gyrus of the hippocampus of adult C57BL/6J mice exposed to the flurothyl kindling model of epileptogenesis. *Neuroscience* 115(3):669-83.
- Goddard GV. 1983. The Kindling Model of Epilepsy. *Trends in Neurosciences* 6(7):275-9.
- Goddard GV, McIntyre DC, Leech CK. 1969. A permanent change in brain function resulting from daily electrical stimulation. *Experimental Neurology* 25(3):295-330.
- Gomez-Lira G, Trillo E, Ramirez M, Asai M, Sitges M, Gutierrez R. 2002. The expression of GABA in mossy fiber synaptosomes coincides with the seizure-induced expression of GABAergic transmission in the mossy fiber synapse. *Experimental Neurology* 177(1):276-83.
- Gray WP, Sundstrom LE. 1998. Kainic acid increases the proliferation of granule cell progenitors in the dentate gyrus of the adult rat. *Brain Res* 790(1-2):52-9.
- Gross CG. 2000. Neurogenesis in the adult brain: death of a dogma. *Nat Rev Neurosci* 1(1):67-73.
- Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A, . 1988. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS* 96(5):379-94.
- Gutierrez R, Heinemann U. 2001. Kindling induces transient fast inhibition in the dentate gyrus--CA3 projection. *Eur J Neurosci* 13(7):1371-9.
- Gutierrez R. 2002. Activity-Dependent Expression of Simultaneous Glutamatergic and GABAergic Neurotransmission From the Mossy Fibers In Vitro. *Journal of Neurophysiology* 87(5):2562-70.
- Hodges H, Katzung N, Sowinski P, Hopewell JW, Wilkinson JH, Bywaters T, Rezvani M. 1998. Late behavioural and neuropathological effects of local brain irradiation in the rat. *Behavioural Brain Research* 91(1-2):99-114.
- Huttman K, Sadgrove M, Wallraff A, Hinterkeuser S, Kirchhoff F, Steinhäuser C, Gray WP. 2003. Seizures preferentially stimulate proliferation of radial glia-like astrocytes in the adult dentate gyrus: functional and immunocytochemical analysis. *European Journal of Neuroscience* 18(10):2769-78.
- Jenrow KA, Ratkewicz AE, Elisevich KV. 2001. Enhanced excitability induced by ionizing radiation in the kindled rat. *Exp Neurol* 169(1):96-104.
- Jenrow KA, Ratkewicz AE, Zalinski DN, Roszka KM, Lemke NW, Elisevich KV. 2006. Influence of ionizing radiation on the course of kindled epileptogenesis. *Brain Res* 1094(1):207-16.
- Jessberger S, Romer B, Babu H, Kempermann G. 2005. Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. *Exp Neurol* 196(2):342-51.

- Jung KH, Chu K, Kim M, Jeong SW, Song YM, Lee ST, Kim JY, Lee SK, Roh JK. 2004. Continuous cytosine-b-D-arabinofuranoside infusion reduces ectopic granule cells in adult rat hippocampus with attenuation of spontaneous recurrent seizures following pilocarpine-induced status epilepticus. *Eur J Neurosci* 19(12):3219-26.
- Kempermann G, Gage FH. 1999. New nerve cells for the adult brain. *Sci Am* 280(5):48-53.
- Kempermann G, Jessberger S, Steiner B, Kronenberg G. 2004. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci* 27(8):447-52.
- Lie DC, Song H, Colamarino SA, Ming GL, Gage FH. 2004. Neurogenesis in the adult brain: new strategies for central nervous system diseases. *Annu Rev Pharmacol Toxicol* 44:399-421.
- Lothman EW, Hatlelid JM, Zorumski CF, Conry JA, Moon PF, Perlin JB. 1985. Kindling with rapidly recurring hippocampal seizures. *Brain Res* 360(1-2):83-91.
- Lothman EW, Williamson JM. 1994. Closely spaced recurrent hippocampal seizures elicit two types of heightened epileptogenesis: a rapidly developing, transient kindling and a slowly developing, enduring kindling. *Brain Res* 649(1-2):71-84.
- Madsen TM, Treschow A, Bengzon J, Bolwig TG, Lindvall O, Tingstrom A. 2000. Increased neurogenesis in a model of electroconvulsive therapy. *Biol Psychiatry* 47(12):1043-9.
- Maru E, Goddard GV. 1987. Alteration in dentate neuronal activities associated with perforant path kindling : II. Decrease in granule cell excitability. *Experimental Neurology* 96(1):33-45.
- McNamara JO. 1984. Kindling - An Animal-Model of Complex Partial Epilepsy. *Annals of Neurology* 16:S72-S76.
- Milgram NW, Michael M, Cammisuli S, Head E, Ferbinteanu J, Reid C, Murphy MP, Racine R. 1995. Development of spontaneous seizures over extended electrical kindling. II. Persistence of dentate inhibitory suppression. *Brain Res* 670(1):112-20.
- Ming GL, Song H. 2005. Adult neurogenesis in the mammalian central nervous system. *Annu Rev Neurosci* 28:223-50.
- Mizumatsu S, Monje ML, Morhardt DR, Rola R, Palmer TD, Fike JR. 2003. Extreme sensitivity of adult neurogenesis to low doses of X-irradiation. *Cancer Res* 63(14):4021-7.
- Mohapel P, Ekdahl CT, Lindvall O. 2004. Status epilepticus severity influences the long-term outcome of neurogenesis in the adult dentate gyrus. *Neurobiol Dis* 15(2):196-205.
- Monje ML, Mizumatsu S, Fike JR, Palmer TD. 2002. Irradiation induces neural precursor-cell dysfunction. *Nat Med* 8(9):955-62.
- Monje ML, Palmer T. 2003. Radiation injury and neurogenesis. *Curr Opin Neurol* 16(2):129-34.
- Monje ML, Toda H, Palmer TD. 2003. Inflammatory blockade restores adult hippocampal neurogenesis. *Science* 302(5651):1760-5.
- Nakagawa E, Aimi Y, Yasuhara O, Tooyama I, Shimada M, McGeer PL, Kimura H. 2000. Enhancement of progenitor cell division in the dentate gyrus triggered by initial limbic seizures in rat models of epilepsy. *Epilepsia* 41(1):10-8.
- Nusser Z, Hajos N, Somogyi P, Mody I. 1998. Increased number of synaptic GABAA receptors underlies potentiation at hippocampal inhibitory synapses. *Nature* 395(6698):172-7.
- Overstreet-Wadiche LS, Bromberg DA, Bensen AL, Westbrook GL. 2006. Seizures accelerate functional integration of adult-generated granule cells. *J Neurosci* 26(15):4095-103.
- Parent JM, Elliott RC, Pleasure SJ, Barbaro NM, Lowenstein DH. 2006. Aberrant seizure-induced neurogenesis in experimental temporal lobe epilepsy. *Ann Neurol* 59(1):81-91.
- Parent JM, Janumpalli S, McNamara JO, Lowenstein DH. 1998. Increased dentate granule cell neurogenesis following amygdala kindling in the adult rat. *Neurosci Lett* 247(1):9-12.
- Parent JM, Tada E, Fike JR, Lowenstein DH. 1999. Inhibition of dentate granule cell neurogenesis with brain irradiation does not prevent seizure-induced mossy fiber synaptic reorganization in the rat. *J Neurosci* 19(11):4508-19.

- Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH. 1997. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci* 17(10):3727-38.
- Peissner W, Kocher M, Treuer H, Gillardon F. 1999. Ionizing radiation-induced apoptosis of proliferating stem cells in the dentate gyrus of the adult rat hippocampus. *Brain Res Mol Brain Res* 71(1):61-8.
- Pierce JP, Melton J, Punsoni M, McCloskey DP, Scharfman HE. 2005. Mossy fibers are the primary source of afferent input to ectopic granule cells that are born after pilocarpine-induced seizures. *Exp Neurol* 196(2):316-31.
- Raber J, Fan Y, Matsumori Y, Liu Z, Weinstein PR, Fike JR, Liu J. 2004. Irradiation attenuates neurogenesis and exacerbates ischemia-induced deficits. *Ann Neurol* 55(3):381-9.
- Racine R, Rose PA, Burnham WM. 1977. Afterdischarge thresholds and kindling rates in dorsal and ventral hippocampus and dentate gyrus. *Can J Neurol Sci* 4(4):273-8.
- Racine RJ. 1972. Modification of seizure activity by electrical stimulation: II. Motor seizure. *Electroencephalography and Clinical Neurophysiology* 32(3):281-94.
- Romcy-Pereira RN, Garcia-Cairasco N. 2003. Hippocampal cell proliferation and epileptogenesis after audiogenic kindling are not accompanied by mossy fiber sprouting or fluoro-jade staining. *Neuroscience* 119(2):533-46.
- Scharfman HE, Goodman JH, Sollas AL. 2000. Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. *J Neurosci* 20(16):6144-58.
- Scharfman HE, Sollas AE, Berger RE, Goodman JH, Pierce JP. 2003. Perforant path activation of ectopic granule cells that are born after pilocarpine-induced seizures. *Neuroscience* 121(4):1017-29.
- Schwarzer C, Sperk G. 1995. Hippocampal Granule Cells Express Glutamic-Acid Decarboxylase-67 After Limbic Seizures in the Rat. *Neuroscience* 69(3):705-9.
- Scott BW, Wang S, Burnham WM, De Boni U, Wojtowicz JM. 1998. Kindling-induced neurogenesis in the dentate gyrus of the rat. *Neurosci Lett* 248(2):73-6.
- Scott BW, Wojtowicz JM, Burnham WM. 2000. Neurogenesis in the dentate gyrus of the rat following electroconvulsive shock seizures. *Exp Neurol* 165(2):231-6.
- Shapiro LA, Korn MJ, Ribak CE. 2005. Newly generated dentate granule cells from epileptic rats exhibit elongated hilar basal dendrites that align along GFAP-immunolabeled processes. *Neuroscience* 136(3):823-31.
- Shapiro LA, Ribak CE. 2005. Integration of newly born dentate granule cells into adult brains: hypotheses based on normal and epileptic rodents. *Brain Res Brain Res Rev* 48(1):43-56.
- Shapiro LA, Ribak CE. 2006. Newly born dentate granule neurons after pilocarpine-induced epilepsy have hilar basal dendrites with immature synapses. *Epilepsy Res* 69(1):53-66.
- Silasi G, az-Heijtz R, Besplug J, Rodriguez-Juarez R, Titov V, Kolb B, Kovalchuk O. 2004. Selective brain responses to acute and chronic low-dose X-ray irradiation in males and females. *Biochemical and Biophysical Research Communications* 325(4):1223-35.
- Smith PD, Mclean KJ, Murphy MA, Turnley AM, Cook MJ. 2005. Seizures, not hippocampal neuronal death, provoke neurogenesis in a mouse rapid electrical amygdala kindling model of seizures. *Neuroscience* 136(2):405-15.
- Snyder JS, Hong NS, McDonald RJ, Wojtowicz JM. 2005. A role for adult neurogenesis in spatial long-term memory. *Neuroscience* 130(4):843-52.
- Tada E, Parent JM, Lowenstein DH, Fike JR. 2000. X-irradiation causes a prolonged reduction in cell proliferation in the dentate gyrus of adult rats. *Neuroscience* 99(1):33-41.
- Tuff LP, Racine RJ, Adamec R. 1983. The effects of kindling on GABA-mediated inhibition in the dentate gyrus of the rat. I. Paired-pulse depression. *Brain Res* 277(1):79-90.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. 2002. Functional neurogenesis in the adult hippocampus. *Nature* 415(6875):1030-4.

Wang S, Kee N, Preston E, Wojtowicz JM. 2005. Electrophysiological correlates of neural plasticity compensating for ischemia-induced damage in the hippocampus. *Exp Brain Res* 165(2):250-60.

Wojtowicz JM. 2006. Irradiation as an experimental tool in studies of adult neurogenesis. *Hippocampus* 16(3):261-6.

Chapter 7

General discussion

NEUROPOTENCY OF ADULT-DERIVED STEM CELLS

There are several ways to define a stem cell, but most definitions include as a requisite the capacity for self-renewal coupled to the potential to generate various types of differentiated progeny (Watt and Hogan, 2000; Clarke and Frisen, 2001). *Totipotent stem cells* have the potential to differentiate to all mammalian cell types and therefore to generate a new organism. The zygote and single blastomeres from a 2-4 cell morula stage are thought to be the only existing totipotent cell types. *Pluripotent stem cells* can generate cells from all three germ layers (endoderm, mesoderm and ectoderm) but no longer the extra-embryonic tissues and therefore cannot generate a new organism. Human and mouse embryonic stem cells (ESC) have pluripotent capacities (Evans and Kaufman, 1981; Martin, 1981). A second source of pluripotent stem cells are derived from mouse (Matsui and others, 1992; Resnick and others, 1992) and human (Shamblott and others, 1998) primordial germ cells (PGCs) and are termed embryonic germ (EG) cells. EG cells are the only cells beyond the gastrulation stage, for which pluripotency has been demonstrated in such a rigorous way, both *in vitro* and *in vivo*. During gastrulation pluripotent cells are destined to become mesoderm, endoderm and ectoderm and subsequently to produce tissue specific cells. Stem cells which generate different tissue-specific cell types are called *multipotent stem cells*, while stem cells that produce only one differentiated cell type are termed *unipotent stem cells*.

A considerable number of reports over the past few years have tackled the general belief that the differentiation potential of adult stem cells is limited by tissue-specific boundaries. These reports describe the ability of stem cells to produce cells of other tissues or even other germ layers. This phenomenon is called *plasticity*. Bone marrow (BM)-derived cells were reported to generate skeletal muscle cells (Ferrari and others, 1998; Bittner and others, 1999; Gussoni and others, 1999), cardiac muscle cells (Orlic and others, 2001), hepatocytes (Lagasse and others, 2000), astrocytes (Eglitis and Mezey, 1997) and neurons (Azizi and others, 1998; Kopen and others, 1999; Brazelton and others, 2000; Mezey and others, 2000; Sanchez-Ramos and others, 2000; Woodbury and others, 2000; Black and Woodbury, 2001; Deng and others, 2001; Kohyama and others, 2001; Priller and others, 2001; Kim and others, 2002; Jiang and others, 2002; Kabos and others, 2002; Mezey and others, 2003; Jiang and others, 2003; Jin and others, 2003; Qian and Saltzman, 2004; Munoz-Elias and others, 2004; Abouelfetouh and others, 2004; D'Ippolito and others, 2004). Hepatic oval cells (Toma and others, 2001) and stem cells derived from adipose tissue (Toma and others, 2001) and skin (Toma and others, 2001) have been differentiated towards neural cell types. Muscle-derived cells have been reported to reconstitute all hematopoietic lineages when transplanted into irradiated mice (Jackson and others, 1999). For neural stem cells (NSC) it was demonstrated that they could generate HSC (Bjornson and others, 1999), muscle cells (Galli and others, 2000; Tsai and McKay, 2000) and endothelial cells (Wurmser and others, 2004). Two adult-derived stem cell types, namely NSC (Clarke and others, 2000) and multipotent adult progenitor cells (MAPC) (Jiang and others, 2002), even contribute to most tissues of the mouse embryo when injected into

mouse blastocysts. In the brain, MAPC-derived cells differentiated towards appropriate region specific neuronal phenotypes but also towards astrocytes and oligodendrocytes (Keene and others, 2003).

Although these reports indicate that some adult-derived cells could be capable for reprogramming and transdifferentiation, cautious interpretation of these apparent *in vitro* and *in vivo* lineage switches is mandatory. Several alternative explanations can be given for the apparent lineage switch.

Firstly, populations of tissue-specific stem or progenitor cells may be more heterogeneous than previously thought and may contain cells with a similar antigenic profile, but with more differentiation potential. In the tissue-specific environment these cells only generate the appropriate cell phenotype, but in other niches they are capable of producing other phenotypes.

Secondly, a mixed cell population derived from specific tissues may contain precursor cells which normally reside in distant organs. For example, muscle-derived cells may contain HSC with the latter being responsible for the repopulation of the hematopoietic system after transplantation of muscle-derived cells in lethally irradiated mice (Jackson and others, 1999).

Thirdly, cell fusion between two cell types originating from different tissues can result into cells with characteristics of one cell type while still expressing markers, specific for the other cell type. BM-derived cells and NSC were shown to spontaneously fuse with ESC, resulting in the adoption of ESC characteristics while still expressing donor specific markers (Terada and others, 2002; Ying and others, 2002). Fused NSC/ESC contributed to multiple lineages of mouse chimeras (Ying and others, 2002).

Fourthly, some studies demonstrating *in vivo* plasticity rely on cell markers which can be transferred upon death of the transplanted cells. 5'-bromo-2'-deoxyuridine (BrdU) or tritiated thymidine labelling of the DNA of proliferating cells and subsequent identification with immunohistochemistry or autoradiography is frequently used. Recently, it has been demonstrated that oligonucleotides from transplanted death cells can be recycled in endogenous proliferating cells or cells undergoing DNA repair (Burns and others, 2006). Similar marker recycling occurs when membrane intercalating dyes are used (Yoon and others, 2005). Preferentially, transgenetically labelled cells are used which for example express GFP or β -galactosidase genes under the control of a tissue specific promoter.

Fifthly, a lot of studies which claim transdifferentiation have based their conclusion solely on morphology and expression of some tissue specific marker genes or proteins. However, care should be taken in the interpretation of this evidence. For example, it has recently been shown that fibroblasts can be induced to adopt neural morphology and express neural antigens when exposed to stressors (Lu and others, 2004; Bertani and others, 2005).

In this study we isolated cells from the BM of adult rats and cultured them according to the protocol described for MAPC (Jiang and others, 2002). Consequently single cell-derived clones with extensive proliferative capacity, similar morphology and a comparable phenotypic expression profile for the investigated markers

(MHC I⁺, MHC II⁺, CD44^{+/dim}) were derived. Since MAPC were reported to be capable of generating cells from the three germ layers (endoderm, ectoderm and mesoderm), several attempts were made to differentiate multiple cell clones towards cells expressing endothelial, hepatocyte or neuroglial markers using the differentiation protocols originally described. RT-PCR and immunocytochemistry were used for the detection of differentiation markers. However, no reproducible results were obtained for any of the lineages. Therefore we termed our cells MAPC-like. Among these MAPC-like cell clones we identified clones which expressed Oct4, a presumed marker for pluripotency.

In order to investigate whether neural transdifferentiation could be optimized we exposed an Oct4 expressing MAPC-like cell clone to four different neural “transdifferentiation” protocols. In order to objectively assess neural transdifferentiation of the MAPC-like cells, we compared morphology and neural antigen expression to that of NSC, subjected to the same differentiation protocols. NSC were obtained by isolating cells from the subventricular zone (SVZ) of adult rats and culturing them as spherical clusters (neurospheres) as described by Gobbel and coworkers (Gobbel and others, 2003). A single cell-derived NSC clone was selected based on its ability to generate cells expressing neuronal, astrocyte and oligodendrocyte markers using a standard NSC differentiation protocol (Reynolds and Weiss, 1996).

The first neuronal transdifferentiation protocol was optimized for neural differentiation of MAPC (Jiang and others, 2002; Jiang and others, 2003). The second protocol was described by Kohyama and colleagues for the differentiation of MSC (Kohyama and others, 2001). The third protocol was described by Kabos and coworkers and reported neural differentiation of unfractionated bone marrow as starting material (Kabos and others, 2002). The fourth protocol used was described by Woodbury and coworkers and was applied for neural differentiation of MSC (Woodbury and others, 2000; Black and Woodbury, 2001; Woodbury and others, 2002). An overview of the used protocols is given in table 1.

Using RT-PCR and immunocytochemistry gene and antigen expression of three neuronal markers (β 3tub, NF200 and tau), an astrocyte marker (GFAP) and two oligodendrocyte markers [MBP (RT-PCR) or RIP (immunocytochemistry)] were evaluated. Marker expression was screened in NSC and MAPC-like cells before and after differentiation.

We found that undifferentiated NSC expressed all five neuroglial genes at baseline. Immunocytochemistry analysis showed that NSC expressed the RIP antigen at baseline but no clear expression of the other markers was found. In three of the four differentiation protocols NSC adopted complex neuronal and glial morphologies, upregulated expression of all five neuroglial genes and stained positive for both the neuronal and glial antigens.

In the Woodbury protocol NSC did not survive differentiation. This finding is in line with recent evidence suggesting that the rapid induction of neural morphology and expression of neural markers in the Woodbury differentiation protocol represents a stress artefact rather than true transdifferentiation (Neuhuber and others, 2004; Lu and others, 2004; Bertani and others, 2005). In these studies time-lapse video recording showed that

the adoption of “neural” morphology was due to a retraction of the cytoplasm while leaving behind long processes. Although these processes strikingly resembled neurites they showed no motility or further elaboration. These studies also demonstrated that similar “neuronal morphology” and expression of neuronal antigens (NeuN, NSE, NF-200, Tau, ...) could be obtained by exposure of MSC but also of other cell types (HEK 293 cells, fibroblasts) to DMSO alone or to other stressors such as actin filament-depolymerising agents, detergents, high molarity sodium chloride and pH extremes.

Table 1: Four protocols for neural differentiation of MAPC-like cells and NSC

	Jiang method	Kohyama method	Kabos method	Woodbury method
Pre-differentiation phase (MAPC-like cells)	7 days: 100 ng/ml bFGF	3 days: 10 µmol/ml 5-aza 50 ng/ml NGF 50 ng/ml BDNF 50 ng/ml NT-3	7 days: 20 ng/ml bFGF 20 ng/ml EGF	1 day: 5 ng/ml bFGF
Differentiation phase (MAPC-like cells and NSC)	7 days: 10 ng/ml FGF-8 100 ng/ml SHH	9 days: 50 ng/ml NGF 50 ng/ml BDNF 50 ng/ml NT-3	7 days: 1 µM RA 1mM db-cAMP	7 days: 100 µM BHA 2% DMSO 10 µM forskolin 2 mM valproic acid 10 mM KCl

Abbreviations: 5-aza, 5-azacytidine; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; BHA, butylated hydroxyanisole; db-cAMP, dibutyryl cyclic adenosine monophosphate; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; FGF, fibroblast growth factor; NGF, nerve growth factor; NT-3, neurotrophin-3; RA, retinoic acid; SHH, sonic hedgehog

In our study, RT-PCR and immunocytochemistry indicated that undifferentiated MAPC-like cells expressed nestin, β3-tub and NF200 at baseline. After differentiation using the three remaining protocols (Jiang, Kohyama and Kabos method), we found that MAPC-like cells extended neurite-like structures and adopted a neural-like morphology only in the Jiang protocol. β3-tub and NF200 were still expressed in differentiated MAPC-like cells but β3-tub expression was downregulated and NF-200 expression was only very sparsely upregulated in two of the three differentiation protocols. Differentiated MAPC-like cells did not express the other neuroglial markers: tau, GFAP, MBP, RIP.

This failure of MAPC-like cells to upregulate or *de novo* express neuroglial markers is in contrast with the successful neural transdifferentiation of MAPC reported by Jiang and colleagues (Jiang and others, 2002; Jiang and others, 2003). The most likely explanation for this incongruence is that the MAPC-like cell clones derived in

our study were substantially different from the MAPC clones originally described by Jiang and colleagues. This hypothesis was further supported by the inability of our MAPC-like cells to generate cells from the three germinal layers and the fact that they expressed neuronal markers at baseline, which was not the case for the MAPC, described by Jiang and coworkers. Most probably the cell lines used in our study were a subtype of MSC with low expression of CD44 and a high expansion capacity. As MSC, our cells could be differentiated reproducibly towards mesenchymal cell types (osteoblasts and adipocytes (Pittenger and others, 1999)).

As already described earlier there have been numerous reports claiming neural transdifferentiation of BM-derived cells, most of them based on the adoption of neural morphology and expression of neuro-glial markers by the differentiated cells. Our study indicates that both parameters should be interpreted with caution. Although we found that MAPC-like cells can adopt neural morphology and express neural markers, a comparison with NSC showed that this did not equal neuropotency. The fact that neural transdifferentiation protocols, proven to work for differentiation of NSC, did not lead to upregulation or *de novo* expression of neural makers in the MAPC-like cells, supported this conclusion.

Apart from the results of our study, there are several lines of evidence from the literature indicating the lack of specificity of morphology change and marker expression. Firstly, reports have shown that cells adopt neural-like morphologies due to the retraction of cytoplasm in response to different stressors (Neuhuber and others, 2004; Lu and others, 2004; Bertani and others, 2005). Secondly, stressors can also induce or enhance the expression of neural markers even in cells with no multilineage potential such as fibroblasts (Lu and others, 2004; Bertani and others, 2005). Thirdly, mature neural genes and antigens such as β 3-tub, NeuN, MAP2, NF-200 are also expressed in hematopoietic progenitor cells (Goolsby and others, 2003) and undifferentiated bone marrow stromal cells (Woodbury and others, 2002; Neuhuber and others, 2004; Tondreau and others, 2004; Ratajczak and others, 2004; Bertani and others, 2005), indicating that these makers are not uniquely expressed in neurons. Fourthly, when different studies, using the same type of stem cells and the same differentiation protocol, are compared for antigen expression before and after differentiation, several inconsistencies are found for different markers (Woodbury and others, 2000; Neuhuber and others, 2004; Lu and others, 2004; Bertani and others, 2005). Fifthly, a recent study showed that it is possible to induce expression of neural specific antigens and neurotransmitters by exposing bone marrow cells to different growth factors mixes. But a closer evaluation showed that the intracellular distribution of the antigens was different from the one seen in adult neurons, implicating that these cells probably cannot function as true neurons (Jin and others, 2003).

Only a limited number of studies have performed electrophysiological analysis on the BM-derived putative neurons (Kohyama and others, 2001; Hofstetter and others, 2002; Hung and others, 2002; Dezawa and others, 2004; Wislet-Gendebien and others, 2005). In two reports neuron-like cells derived from MSC lacked not only voltage-gated Na⁺ and K⁺ currents and action potentials, but also functional neurotransmitter receptors (Hofstetter and others, 2002; Padovan and others, 2003). Other reports showed that MSC do acquire some electrical properties of functionally active neurons (Kohyama and others, 2001; Hung and others, 2002; Dezawa and others, 2004; Wislet-Gendebien and others, 2005).

Kohyama and coworkers recorded outward rectifying potassium currents and increased Ca^{2+} uptake in response to neurotransmitters and high extracellular potassium from the differentiated MSC. However, no voltage-gated fast sodium currents were measured and the resting membrane potentials in the cells were higher compared to normal functional neurons (Kohyama and others, 2001).

Hung and coworkers differentiated MSC using retinoic acid (RA) and β -mercaptoethanol and could record voltage dependent currents from these differentiated MSC five days after differentiation. They also showed Ca^{2+} uptake in response to glutamate or high K^+ buffer but the observed elevation in intracellular Ca^{2+} was either slow or delayed (Hung and others, 2002).

Wislet-Gendebien and colleagues reported that nestin-positive MSC, co-cultured with cerebellar granule neurons, had a resting membrane potential of -57 mV and inward sodium currents after 15 days of co-culture. Ionic currents in response to neurotransmitters (GABA, glutamate and glycine) were measured and single action potentials evoked. However trains of action potentials were not evoked nor was spontaneous synaptic activity detected indicating that the cells were still in an intermediate phase of neuronal development. The authors excluded the possibility that these results were due to cell fusion of MSC with cerebellar granule neurons by showing that similar results were obtained when MSC were co-cultured with formalin-fixed granule neurons in the presence of granule cell conditioned medium (Wislet-Gendebien and others, 2005).

Dezawa and colleagues overexpressed the intracellular domain of Notch in MSC and subjected them to a neural differentiation protocol using forskolin, bFGF, ciliary neurotrophic factor (CNTF), BDNF and NGF. Electrophysiological analysis showed the presence of both outward rectified potassium current and inward fast sodium currents. Also action potentials could be evoked in these differentiated MSC. Exposure of the differentiated MSC to GDNF resulted in more than 40% of the cells expressing tyrosine hydrolase (TH) suggestive of dopaminergic commitment. Further evidence for dopaminergic-like neuronal differentiation resulted from studies in which K^+ depolarization resulted in the release of 1.1 pmol dopamine/ 10^6 cells and improvement of the rotational behaviour of a rat model for Parkinson's disease to a similar extent as described for fetal midbrain transplantation (Dezawa and others, 2004).

Taken together, there is a large body of evidence that BM-derived cells can acquire neural-like morphologies and express neural antigens *in vitro*. But our own study, together with others, has shown that both parameters are too unspecific to validate neural transdifferentiation of adult non-neural cells. There have been electrophysiological studies showing that MAPC but also MSC can generate cells with functional properties of immature neurons but this requires complex differentiation protocols involving the use of genetic engineering or coculture with astrocytes or mature neurons. Based on our own results and the conflicting reports in literature on neuropotency of BM-derived stem cells, we chose to transplant adult-derived NSC in a model for TLE.

CHRONIC MODEL FOR TEMPORAL LOBE EPILEPSY: THE INTRAHIPPOCAMPAL KAINIC ACID MODEL

The intrahippocampal kainic acid (KA) model is a chronic model for temporal lobe epilepsy (TLE) that consists of the injection of a small amount of KA, a glutamatergic analogue. Injection of KA has excitotoxic effects, causing a local lesion and the induction of status epilepticus (SE). As in other SE models, the intrahippocampal KA model develops highly comparable neuropathological alterations as to human TLE patients. These alterations include hippocampal sclerosis, extensive mossy fiber sprouting and dispersion of the GCL (Schwarcz and others, 1978; French and others, 1982; Cavalheiro and others, 1982; Leite and others, 1996; Riban and others, 2002).

Intracranial injection of KA in anaesthetized rats results in spontaneous recurrent seizures occurring at a low frequency only in a small proportion of animals. This is most likely due to the anti-epileptogenic effects of the anaesthesia used during local KA injection. For example, Bragin and coworkers injected 20 anaesthetized rats in the right posterior hippocampus with 0.4µg/0.2µl KA. Rats underwent 4 weeks of video-EEG monitoring from 3 to 8 months after KA treatment but seizures were only detected in 8 out of 20 rats (40%) (Bragin and others, 1999b). In a follow-up study, the same group injected KA in unanaesthetized rats and seizures were detected in 73% of the rats during the first three months after KA injection (Bragin and others, 2004).

In analogy to human patients, Bragin and coworkers detected aberrant high frequency oscillations (HFO's) in the EEG recorded from the dentate gyrus ipsilateral to the intrahippocampal KA injection (Bragin and others, 1999a). These HFO's consisted of ripples (100-200 Hz) and fast ripples (200-500 Hz) and only occurred in rats that developed spontaneous seizures later on. The latency to the first HFO was positively correlated with the latency to the first seizure detection and the frequency of spontaneous seizures (Bragin and others, 2004). Therefore, HFO's are considered as pathological and probably epileptogenic. Bragin and colleagues also found two seizure onset types in this model which are also described in human patients. A large fraction (74%) of the seizures started locally in the dentate gyrus ipsilateral to the KA injection, with an increase in frequency of interictal EEG spikes (hypersynchronous type) and 26% of seizures started bilaterally with a decrease of EEG amplitude and parallel increase in frequency (low-voltage fast type) (Bragin and others, 2005).

Despite detailed anatomopathological characterization and electrophysiological description of interictal and ictal phenomena, habitual seizure parameters such as seizure frequency, duration and severity were not yet characterised for the intrahippocampal KA model. Therefore, we monitored SE and subsequent spontaneous seizures in this model using video-EEG monitoring. We found that SE and spontaneous seizure characteristics in this model were very similar to those in other SE models for TLE (table 2).

As reported by Bragin and others, we found that intrahippocampal KA injection through a cannula in unanaesthetized rats induced a SE in every rat (Bragin and others, 2004). Therefore, intrahippocampal KA injection represents the most efficient among all methods to evoke a SE (table 2). The fraction of rats developing SE after intraperitoneal injection of KA is 93% (Stafstrom and others, 1992), after intraperitoneal PILO injection 61-73% (Gliem and others, 2001), after intrahippocampal injection of PILO 71% (Furtado Mde and

others, 2002). Continuous stimulation of the hippocampus evokes SE in 70% (Bertram and Cornett, 1994), of the perforant path in 71%-100% (Mazarati and others, 1998; Gorter and others, 2001) and of the amygdala in 87% of the rats (Nissinen and others, 2000).

In our study all rats survived SE although we needed to terminate SE in two rats to prevent possible death of the animals. The highest mortality was seen after systemic injection of a single bolus of KA or PILO with can reach up to 50% (Hellier and others, 1998) and 40 % (Gliem and others, 2001) respectively (table 2). However, injection of repeated low doses with individual adjustments of the dose highly reduces mortality to respectively 15% and 7 % (Hellier and others, 1998; Gliem and others, 2001). In the models in which SE is evoked by continuous electrical stimulation mortality was highest after stimulation of the amygdala (20%) (Nissinen and others, 2000). Continuous stimulation of the hippocampus or the perforant path induces SE without causing mortality (Bertram and Cornett, 1994; Mazarati and others, 2002).

The duration of SE, measured on the EEG, after intrahippocampal KA injection was about 10 hours. In many studies, using other SE models, electrographic duration of SE could not be determined because SE was terminated by pentobarbital or diazepam injection to limit variability and/or mortality (Gorter and others, 2001; Gliem and others, 2001; Furtado Mde and others, 2002) or due to the absence of EEG recording (Stafstrom and others, 1992; Hellier and others, 1998). Studies that did determine the electrographic duration of SE showed that this was comparable to our findings despite the use of other methods for its induction (table 2). The duration of SE induced by continuous hippocampal stimulation varied between 6 and 12 hours (Bertram and Cornett, 1994), SE after continuous perforant path stimulation lasted for 9 h (Mazarati and others, 2002) and in the continuous amygdala stimulation model for about 12 hours (Nissinen and others, 2000).

Table 2: Comparison of the intrahippocampal KA model with other SE models for human TLE

Ref.	Rat strain (age)	Induction (dose)	Rats with SE	SE duration (EEG)	Rats with SRS (monitoring period)	SRS onset (d)	Partial SRS	SRS duration	Rats with frequent SRS ($\geq 3/d$)
Raedt (2007)	SPRD (200-220g)	single KA (0.4μg/0.2μl, HC)	100%	10 h	87% (21 wks; 72h/wk)	28 (7-63)	34%	64 \pm 4 s	3 of 15 rats (20%)
Hellier (1998)	SPRD (150-250 g)	multi KA (5 mg/kg, ip)	n.d.	n.d.	97% (28 wks; 6-8h/wk)	77 \pm 38	n.d.	n.d.	n.d.
Stafstrom (1990)	SPRD (60d)	single KA (10-12 mg/kg, ip)	93%	n.d.	44% (3 months; n.d.)	34 (4-121)	n.d.	40 s (video)	n.d.
Glenn (2001)	Wistar (200-225 g)	single PILO (30 mg/kg, ip)	73%	interrupted	78% (19 wks; 12-56h/wk)	12-72	n.d.	27 s (video)	n.d.
Glenn (2001)	Wistar (200-250g)	multi PILO (10 mg/kg, ip)	61%	interrupted	100% (19 wks; 12-56h/wk)	10-75	n.d.	27 s (video)	n.d.
Furtado (2002)	Wistar (200-250 g)	single PILO (2.4 μ g/ μ l, HC)	71%	interrupted	71% (30 days; 8h/d)	2-30	16%	n.d.	n.d.
Bertram (1994)	SPRD (200-225g)	90 min stim (400 μ A) in HC	70%	6-12 h	100% (18 wks; continuously)	n.d.	n.d.	64-106 s	n.d.
Gorter (2001)	Wistar (350-550 g)	90 min stim (500 μ A) in PP	71%	interrupted	100% (6 m, continuously)	7.2-8.6	n.d.	60 s	10 of 15 rats (67%)
Mazarati (2002)	Wistar (12-14wk)	30 min stim (20V) in PP	100%	9 h	n.d.	20-32	n.d.	120 s	13 of 17 rats (76%)
Nissinen (2000)	SPRD (320-390 g)	20-30 min stim (400 μ A) in AM	87%	12h	87% (26 wks; 24h/d; 3.5d/wk)	28 (6-85)	21%-77%	49 s	4 of 13 rats (31%)

Abbreviations: AM, amygdala; d, day; HC, hippocampus; ip, intraperitoneal; KA, kainic acid; min, minute; n.d., not determined; PILO, pilocarpine; PP, perforant path; Ref, references; SE: status epilepticus; SPRD, Sprague Dawley; SRS, spontaneous recurrent seizures; stim, stimulation; wk, week

We detected spontaneous seizures in 13 out of 15 animals (87%) during the first 21 weeks after SE, with all these rats having their first seizure in the first eight weeks after SE. In the remaining two rats seizures were detected during the second monitoring period 40 to 42 weeks after induction of SE. Similar high numbers of animals developing spontaneous seizures were reported in other SE models. Intraperitoneal injection of a single dose or multiple doses of PILO induced spontaneous seizures in respectively 78% and 100 % of the injected animals (Glien and others, 2001). After intraperitoneal injection of multiple small doses of KA, 97% of the animals developed spontaneous seizures (Hellier and others, 1998). SE evoked by continuous stimulation in the hippocampus or in the perforant path resulted into spontaneous seizures in all treated rats (Gorter and others, 2001; Mazarati and others, 2002), while after continuous amygdala stimulation spontaneous seizures were detected in 87% of the animals (Nissinen and others, 2000). When comparing these studies, it is noted that most studies, including ours, did not continuously monitor the rats and that the number of weeks or months of monitoring varied among the different studies (table 2). Hence, rare seizures could have been missed leading to an underestimation of the fraction of epileptic rats.

An important characteristic of acquired epilepsy is the delay between the initial brain insult and the onset of spontaneous seizures, the so called “latent period”. Determination of the latent period can only be performed when rats are monitored continuously (24h/day, 7 days/week). A study by Gorter and colleagues reported the first spontaneous seizure after a mean latent period of only one week in a model where SE was induced by continuous perforant path stimulation (Gorter and others, 2001). In the other models, the duration between SE and the first seizure detection was higher, most likely due to the fact that animals were monitored only during a fraction of time. In our study, rats were monitored for 72 hours per week and the time between SE and the first seizure detection varied between 7 and 63 days. In studies which monitored post-SE rats for similar fractions of time, the duration between SE and the first seizure detection was comparable (table 2): after intraperitoneal injection of PILO the first seizure was detected 10 to 75 days after SE (Glien and others, 2001), after continuous amygdala stimulation the first seizure was detected 6 to 85 days after SE (Nissinen and others, 2000). In the study done by Hellier and colleagues rats were monitored for only 6 to 8 hours per week. In this study the mean duration for the first seizure detection was longer: 77 days (Hellier and others, 1998).

We found that 34% of the spontaneous seizures were partial without secondary generalization (SG). Only a few other studies recorded simultaneously video and EEG which is mandatory to determine the number of seizures that evolve to SG (table 2). In the intrahippocampal PILO model, 16% of the partial seizures did not secondary generalize (Furtado Mde and others, 2002). In the continuous amygdala stimulation model 21% of the partial seizures do not show SG during the first 10 weeks of monitoring but this increased up to 77% in the following weeks (Nissinen and others, 2000).

The mean seizure duration varied between 27 and 120 seconds among models (table 2). In studies which determined duration of seizures based on video-images, the average seizure duration ranged between 27 and 40 seconds (Stafstrom and others, 1992; Glien and others, 2001). In studies using EEG to determine seizure

length, seizure duration varied from 49s to 106s (Bertram and Cornett, 1994; Nissinen and others, 2000; Gorter and others, 2001). We determined seizure duration based on the EEG and found that this was on average 64s.

As described in other SE models, seizure frequency was highly variable among rats (table 2). We found that three of the 15 rats (20%) had frequent seizures, while 73% of the animals had a low seizure frequency with less than one seizure per day. This dichotomy in frequently and rare seizing rats has also been described for the continuous amygdala and perforant path stimulation models. In the continuous amygdala stimulation model, 31% of the animals displayed frequent seizures (Nissinen and others, 2000). In the perforant path stimulation model, the proportion of rats with frequent seizures was higher, being 67% to 76% (Gorter and others, 2001; Mazarati and others, 2002).

We found that the frequency of convulsive seizures increased over time during the first 20 weeks after SE. Also rats that were monitored a second time 40 weeks after SE all showed a higher seizure frequency compared to the first monitoring session. This shows that the epileptic state in the intrahippocampal KA model is progressive. An increase in seizure frequency over time has also been reported in all other SE models (Bertram and Cornett, 1994; Hellier and others, 1998; Arida and others, 1999; Nissinen and others, 2000; Gorter and others, 2001). Gorter and coworkers identified a subgroup of rats in which progression of seizure frequency was most evident and showed that this progression was associated with loss of hilar interneurons combined with mossy fiber sprouting (Gorter and others, 2001). The progression of seizure frequency in our and other models parallels the situation in most human patients suffering from TLE in whom a worsening of the epileptic state over time has been described (Engel, 1996; Wieser, 2004).

Monitoring for 24 hours/day showed that seizures followed a circadian pattern and occurred more frequently during the day (lights on). Overall $62 \pm 1\%$ of the seizures occurred during the day. The same tendency was found in the intraperitoneal KA models and in the continuous amygdala and hippocampus stimulation models where also about 60 % of the seizures occurred during the day (Hellier and others, 1998; Quigg and others, 1998; Nissinen and others, 2000). Hellier and coworkers normalized seizure occurrence for the activity-state of the animal and they found that more seizures occur during periods of inactivity (82%) compared to periods of activity (18%) (Hellier and others, 1998).

Taken together, by performing an extensive characterization of the intrahippocampal KA model, using video-EEG monitoring, we found that a high proportion of treated rats became epileptic and that seizure parameters resembled those of other post-SE TLE models. Intrahippocampal KA injection has the disadvantage of being more difficult and time consuming than single systemic injection of proconvulsants. However, for the screening of efficacy of focal treatments (such as cell transplantation) this model has the advantage that initial injury and subsequent epilepsy is also focal with a large number of partial seizures without SG and the presence of the seizure focus in the hippocampus ipsilateral to the KA injection (Nadler, 1981; Bragin and others, 1999b; Bragin and others, 2005; Dudek and others, 2006). The high fraction of epileptic rats combined with focal injury and seizure origin motivated us to use this TLE model for the evaluation of cell transplantation.

CELL TRANSPLANTATION IN THE INTRAHIPPOCAMPAL KAINIC ACID MODEL

As described extensively in the introduction, cell transplantation in TLE can be done for two purposes. Firstly, cells can be transplanted in order to replace lost cells and repair damaged circuitry. Secondly, cells can be grafted for the local delivery of seizure-suppressing substances.

CELL TRANSPLANTATION FOR THE REPAIR OF DAMAGED CIRCUITRY

As hippocampal sclerosis is believed to play a major role in the pathogenesis and/or aggravation of TLE and as it is characterised by the loss of hippocampal neurons, replacement of lost hippocampal neurons and repair of hippocampal circuitry may represent a cure for TLE. In the review on cell therapy in models for TLE, presented higher in this dissertation, we listed a number of studies which transplanted fetal hippocampal neurons in the sclerotic hippocampus of an intracerebral KA model for TLE. Part of the transplanted cells formed appropriate synaptic connections and in some cases a partial reversal of anatomopathological changes such as mossy fiber sprouting and loss of GAD immunoreactivity was demonstrated (Shetty and Turner, 1997; Shetty and others, 2000). However two major issues arose from these studies. Firstly, successful results were only obtained when homotopic transplantation was performed, which means that the appropriate cell type needs to be transplanted directly at the correct location (Shetty and Turner, 1997; Shetty and others, 2000; Zaman and others, 2000). Secondly, the transplanted fetal cells did not migrate but remained clumped at the grafted site (Turner and Shetty, 2003). Both issues are important disadvantages for the repair of hippocampal sclerosis which is characterized by widespread loss of different hippocampal cell types. Structural repair of hippocampal sclerosis would require a high amount of injections on different locations throughout the hippocampus, probably resulting in extended damage rather than repair.

The need of high number of fetuses and the inability to obtain a purified population of transplantable cells imply important ethical and practical limitations for the use of fetal tissue for cell based repair. These limitations have urged the search for alternative cell sources to replace fetal tissue. As discussed more extensively in both reviews in the introduction immortalized neural progenitor cells and neuropotent stem cells are promising alternative cell sources.

At least two immortalized progenitor cell lines have been reported to generate different neuronal phenotypes in the adult hippocampus: the RN33b and MHP36 cell line. Both cell lines are able to differentiate towards functionally active hippocampal neurons upon transplantation in the adult hippocampus (Shihabuddin and others, 1995; Englund and others, 2002). After transplantation in the damaged hippocampus, RN33b cells only differentiated towards neurons in regions where they could intermingle with spared host neurons, indicating that differentiation of RN33b cells into specific neuronal phenotypes is not autonomous but depends strongly on the presence of local cues (particularly the direct cell to cell interaction) (Shihabuddin and others, 1996). In contrast to RN33b cells, MHP36 cells migrate towards lesioned brain areas and seem to replace, at least in part, lost neurons. In the 4-VO model intrahippocampally transplanted MHP36 cells migrate to and repopulate the

CA1 region, which is selectively lesioned after 5-15 minutes of global ischemia. A fraction of the MHP36 cells display pyramidal morphology and express neuronal markers. Electrophysiological studies to demonstrate the functionality of these new neurons have not been performed.

Neuropotent stem/progenitor cells can be derived from ESC or isolated directly from CNS tissue. Neural progenitors derived from tau-eGFP ESC were grafted into the mature hippocampus of both normal and chronically epileptic rats. In both cases GFP positive ESC-derived neurons remained clustered close to the injection site without evidence of functional cell replacement (Ruschenschmidt and others, 2005). A commonly reported problem with ESC transplantation is the frequent formation of teratocarcinomas by undifferentiated ESC which were co-transplanted in the brain (Bjorklund and others, 2002; Wernig and others, 2004).

Isolating neural stem/precursor cells directly from CNS tissue avoids this issue. A high number of papers have been published on various protocols for the isolation and expansion of NSC from a variety of regions of both the developing and adult brain of different mammals, including humans (Gage, 2000). Especially the adult-derived NSC are very interesting potential cell sources for the replacement of lost neurons as there are no ethical issues related to their use and it would make autologous transplantation strategies possible.

Based on the successful *in vitro* differentiation results of adult SVZ-derived NSC we chose to use these cells for transplantation in the sclerotic hippocampus. We isolated NSC from the SVZ of adult GFP reporter mice and expanded them as neurospheres for at least 10 passages. After labelling with BrdU, GFP positive NSC (140.000 cells) were transplanted in intact and sclerotic hippocampi of control and KA treated rats respectively. In this latter, NSC were transplanted three days or three weeks after induction of the KA lesion. We found that a limited fraction (1%) of the transplanted cells survived three and six weeks after transplantation both in the intact and sclerotic hippocampus. In both cases a small fraction of the GFP⁺ transplanted cells expressed the neuronal marker NeuN. In the intact hippocampus a maximum of 6% of the GFP⁺ transplanted cells expressed NeuN, while in the sclerotic hippocampus a maximum of 4% of the transplanted cells expressed the neuronal marker. A much larger fraction of the transplanted, GFP⁺ cells expressed GFAP both in the intact hippocampus (30-40%) and the sclerotic hippocampus (50-60%).

It is most likely that the GFP-labeled cells expressing NeuN or GFAP are the result of direct differentiation of the transplanted NSC. However, recent studies have indicated that transplanted stem cells are able to fuse with recipient cells, resulting in cells which express the donor-specific label and display phenotypic properties (morphology, marker expression) of the endogenous cells they fused with (Alvarez-Dolado and others, 2003; Wang and others, 2003). *In vitro*, NSC have been shown to spontaneously fuse with other NSC (Chen and others, 2006; Jessberger and others, 2007) and with ESC (Ying and others, 2002).

We hypothesize that if fusion would have occurred in these transplantation experiments it would probably be at a very low rate based on two arguments. Firstly, cell fusion only rarely occurs both *in vitro* (Terada and others, 2002; Ying and others, 2002; Chen and others, 2006; Jessberger and others, 2007) and *in vivo* (Alvarez-Dolado and others, 2003; Harris and others, 2004). Secondly, Corti and coworkers investigated the possibility of

cell fusion between transplanted NSC and endogenous brain cells by transplanting NSC, derived from female β -actin GFP transgenic mice into male recipients (Corti and others, 2005). Fluorescence in situ hybridization (FISH) for the Y-chromosome did not reveal the presence of a Y-chromosome in any of the investigated (n=2340 cells) GFP labeled cells, indicating that stable cell fusion of NSC with endogenous brain cells was either extremely rare or did not occur (Corti and others, 2005).

Our study setup does not allow to investigate the possibility of cell fusion. In order to do so we need to transplant NSC derived from female β -actin GFP transgenic mice into normal and sclerotic hippocampus of male rats and perform FISH for the rat Y chromosome. Alternatively, we could transplant cells from Z/EG donor mice into mice ubiquitously expressing Cre recombinase. Cells from Z/EG reporter mice express β -galactosidase but when they fuse with Cre expressing cells expression of β -galactosidase switches to GFP expression (Harris and others, 2004).

In the intact hippocampus, donor-derived expressing NeuN were found in the GCL, the zone of active hippocampal neurogenesis, but not in other hippocampal regions (CA1, CA3 or hilus). Other studies which transplanted rodent NSC derived from the SVZ (Richardson and others, 2005; Muraoka and others, 2006), the hippocampus (Gage and others, 1995; Suhonen and others, 1996) or the spinal cord (Shihabuddin and others, 2000) in the hippocampus also found NeuN expressing cells only in the GCL. This indicates that the presence of a neurogenic environment is very important in order for stem cells to acquire a neuronal phenotype. This hypothesis is further strengthened by studies which transplanted the same type of NSC in both neurogenic (dentate gyrus, olfactory bulbs, SVZ) and non-neurogenic CNS regions (striatum, spinal cord, cerebellum). In these studies donor-derived neurons were only found in the neurogenic regions (Suhonen and others, 1996; Shihabuddin and others, 2000; Richardson and others, 2004; Richardson and others, 2005).

Only 6% of the transplanted cells expressing NeuN was low but comparable to another study which transplanted SVZ-derived NSC in the adult hippocampus (Muraoka and others, 2006). This latter study showed that the fraction of cells expressing neuronal markers could be enhanced by performing autologous transplantation. Besides the interspecies barrier, another factor which could have contributed to the low rate of neuronal differentiation may be the culture of the NSC as neurospheres. It has been demonstrated that rodent-derived neurospheres lose their neurogenic potential over time (Winkler and others, 1998; Sun and others, 2003). A recent study cultured SVZ-derived NSC as monolayers instead of as neurospheres and obtained a higher fraction (about 27%) of NSC differentiating towards neuronal cells after grafting in the hippocampus (Richardson and others, 2005). However, this study used BrdU as a single marker and did not exclude the possibility that BrdU was transferred from dying transplanted cells to endogenous dividing granule cell precursors.

In the sclerotic hippocampus, a maximum of about 4% of the surviving transplanted cells had differentiated towards neurons. These donor-derived neurons did not integrate in the GCL but remained within the cluster of transplanted cells. This might indicate that neurogenic signals were still present but that the mechanism

responsible for proper integration of new neurons was no longer functional. This would also explain why some of the endogenous newborn granule neurons are found at abnormal locations in the hilus or molecular layer in the sclerotic hippocampus (Scharfman and others, 2000).

Based on this limited fraction of neuronal differentiation and the location within the transplanted cell cluster there is little or no indication that transplanted NSC replaced lost hippocampal neurons or repaired hippocampal circuitry.

Sofar, reports demonstrating that NSC can generate neurons in damaged brain region are very scarce (Conti and others, 2006; Martino and Pluchino, 2006). Most successful results were obtained by using human fetus-derived NSC. In one study, human fetal NSC were transplanted in the rat brain near the ischemically damaged striatum. Following transplantation, the NSC migrated towards the lesion where the majority of the NSC differentiated towards neuronal cells (Kelly and others, 2004). Similarly, human fetal NSC generated a small fraction of dopaminergic neurons upon transplantation in the striatum of Parkinsonian rats (Svendsen and others, 1997).

To our knowledge, no studies have yet reported replacement of lost pyramidal neurons or hilar interneurons by NSC, grafted in a sclerotic hippocampus. One study injected β -galactosidase labelled human fetal NSC in the tail vein of rats one day after PILO-induced SE. Six weeks later cells were found in the hippocampus, amygdala and pyriform cortex. About 20 % of the cells expressed GFAP and 30% expressed GABA and parvalbumin, two markers for inhibitory hilar interneurons (Chu and others, 2004). Surprisingly, those presumed interneurons did not express the pan-neural markers, NeuN or β 3tubulin, indicating that they were not neurons. Despite the lack of neuronal repair, the authors did find that the fraction of animals which developed spontaneous seizures in the six weeks after PILO induced SE was lower after transplantation. Furthermore, the transplanted animals that did develop spontaneous seizures had a significantly lower seizure frequency, less severe seizures and demonstrated less aggressive behaviour compared to non-transplanted rats.

This latter report is one out of many studies which show that transplantation of NSC induces behavioural improvement without evidence that the transplanted NSC give rise to functionally integrated neurons with significant replacement of lost cells. It seems that transplantation of NSC can also induce “bystander” effects which in their turn can affect disease progression (Martino and Pluchino, 2006). One of these bystander effects is the release by NSC of neurotrophins such as NGF, BDNF, CNTF and GDNF which could result in increased survival and function of endogenous brain cells which survived the initial brain insult (Lu and others, 2003; Yan and others, 2004; Ryu and others, 2004). Another bystander effect is immunomodulation and alteration of the inflammatory environment after transplantation of NSC. For example, it has been shown that transplanted NSC induce apoptosis of T-lymphocytes by the upregulation of membrane expression of certain death receptor ligands (such as FASL, TRAIL and APO3L) (Pluchino and others, 2005) and hamper infiltration of blood-born monocytes at the lesion border of ischemic areas where the NSC accumulate (Kelly and others, 2004).

Taken together, neural stem or precursor cells derived from ESC or primary CNS tissue do not seem to replace neurons and/or mediate circuitry repair in a sclerotic hippocampus. To achieve this, probably other cell sources and/or approaches will be required. If we would succeed to generate cells which replace one or more hippocampal phenotypes in a sclerotic hippocampus a crucial next step would be to make sure that the grafted neurons make appropriate synaptic contacts in order to restore the deregulated balance between excitation and inhibition. It is likely that the introduction of new excitatory neurons in a sclerotic and hyperexcitable hippocampus could have aggravating effects if they are not “connected” in an appropriate way. This need for appropriate integration was already obvious from early transplantation reports which show that intrahippocampal grafting of fetal hippocampal neurons can have pro-epileptic effects with induction of epileptic spikes and occasional behavioural seizures (Buzsaki and others, 1988; Buzsaki and others, 1991).

Another factor complicating neural repair in case of hippocampal sclerosis could be the extensive gliosis. As the hippocampus is highly structurally organized in different cell layers, migration of the transplanted cells towards appropriate location will be required. However, the astrocytic scar forms a barrier for migrating cells. In our study, we found that the dispersion of the transplanted NSC was two times higher in intact hippocampus compared to sclerotic hippocampus. This is most probably a reflection of the presence of gliotic scar tissue.

CELL TRANSPLANTATION FOR LOCAL DELIVERY OF ANTI-EPILEPTIC SUBSTANCES

In a less complex, but until now more successful, alternative approach cells are transplanted for the local delivery of inhibitory substances in epileptic brain structures, responsible for the generation and/or spreading of the seizures. Originally, fetal brain tissue was isolated from regions containing high amounts of seizure suppressing neurotransmitters, such as GABA (striatum), noradrenaline (locus coeruleus) and acetylcholine (basal forebrain) and transplanted. For a detailed overview of these different transplantation studies we refer to the introduction of this dissertation. Because of the practical and ethical limitations of fetal cell transplantation also non-fetal cells were engineered to secrete antiepileptic substances, such as adenosine and GABA.

Adenosine kinase, an adenosine-metabolizing enzyme, was disrupted in fibroblasts (Huber and others, 2001), myoblasts (Guttinger and others, 2005b) and embryonic stem cells (Fedele and others, 2004; Guttinger and others, 2005a). Consequently these cells release high amounts of adenosine. These adenosine-secreting cells were encapsulated in semi-permeable polymers and transplanted in the lateral ventricles of fully kindled rats. After transplantation of the encapsulated cells a temporary but complete suppression of kindling seizures was found.

Thompson and colleagues engineered a conditionally immortalized mouse neuronal cell line (CN1.4) to deliver GABA by driving GAD₆₅ expression using a doxycycline controlled promoter. Grafting of these cells into the anterior SNr (Thompson and others, 2000; Thompson and Suchomelova, 2004), the pyriform cortex (Gernert

and others, 2002) and the dentate gyrus of the hippocampus (Thompson, 2005) of rats showed clear antiepileptic effects.

A common reported problem for the adenosine- and GABA-releasing cells was the inherent inability to survive in the brain upon transplantation which made that the antiepileptic effects were only short-lasting. Even control cells, which were not engineered to overexpress GABA or adenosine, did not survive for a long time after transplantation.

We found that a fraction of the adult-derived NSC survived for at least six weeks after transplantation in both the intact and damaged hippocampus. Therefore adult-derived NSC may represent an alternative cell source for the long-term delivery of antiepileptic substances. However more studies are necessary to increase the fraction of surviving cells. In our study only about 1% of the transplanted NSC seemed to survive. This was rather low compared to a study done by Gage and coworkers, in which it was reported that 41% of the hippocampus-derived NSC survived after homotopic transplantation in the adult hippocampus (Gage and others, 1995). Several explanations are possible for the lower survival rates found in our study.

Firstly, we heterotopically transplanted SVZ-derived NSC in the hippocampus. In case of fetal cell grafting, heterotopic transplantation also led to lower survival rates (Zaman and others, 2000).

Secondly, we xenotransplanted mouse cells into a rat brain. A recent study demonstrated that allogenic transplantation of SVZ-derived NSC resulted in significantly less survival compared to autologous transplantation (Muraoka and others, 2006). The interspecies barrier could be an important factor for the lower survival even with suppression of the immune rejection by cyclosporine.

Thirdly, we cultured the NSC as neurospheres. Neurospheres are heterogeneous clusters which contain NSC but also more differentiated cells. In contrast, monolayer culture seems to allow a more selective expansion of NSC (Conti and others, 2005). In the study done by Gage and colleagues the hippocampal stem cells were expanded as monolayers (Gage and others, 1995). So, the higher number of surviving neurons may represent a higher fraction of NSC in culture.

Fourthly, in our study we might have underestimated cell survival by only counting the cells that double-labelled for BrdU and GFP. However, we found that a considerable fraction of cells were positive for BrdU but not for GFP. Gage and coworkers transplanted NSC which were double-labelled with BrdU and a transgenic marker, in this case β -galactosidase (Gage and others, 1995). As in our study, the authors found that the fraction of transgenetically-labelled cells was lower than the fraction of BrdU labelled cells. However in this study the number of BrdU labelled cells was counted and it was stated that the expression of the transgene was downregulated. However, as already stated above, BrdU can be transferred from dying cells to endogenously dividing cells resulting in false positive results (Burns and others, 2006).

In our study, we could not demonstrate differences in the survival of transplanted cells in a normal or sclerotic hippocampus. We did find that cell survival in a sclerotic hippocampus was significantly higher when cells were

transplanted early (three days) after making the KA lesion compared to transplantation after a longer postlesion delay (three weeks). The same was found in case of homotopic fetal hippocampal cell transplantation. Fetal CA3 cells transplanted early (4 days) after the lesion survived two to four times more compared to fetal cells transplanted 45 days after induction of the KA lesion (Zaman and others, 2001; Turner and Shetty, 2003). The authors speculate that this is due to the enhanced neurotrophic factor environment that is present up to 14 days after lesion development (Lowenstein and others, 1993). In line with their hypothesis, the authors pre-treated the cells before transplantation for three hours with growth factors (bFGF, BDNF, NT-3) and an anti-apoptotic substance (the caspase inhibitor Ac-YVAD-cmk). Because of this pre-treatment, survival rates were increased up to 99%, even when cell were transplanted with a postlesion delay of 4 months (Zaman and Shetty, 2002; Zaman and Shetty, 2003; Hattiangady and others, 2006).

Our study showed that a high fraction of the transplanted cells expressed GFAP and displayed an astrocytic morphology indicating that they had differentiated towards astrocytes. Compared to the intact hippocampus, the fraction of cells acquiring an astrocytic phenotype was enhanced in the sclerotic hippocampus. Moreover, it was evident that the transplanted cells adopted a morphology resembling reactive astrocytes with thick processes, an enlarged cell body and intense staining for GFAP. Both findings indicate that transplanted NSC were recruited in the host astrogliotic response to the lesion.

Recent experiments have indicated that astrocytes could play a prominent role in the generation and/or spreading of seizures by the release of glutamate (Tian and others, 2005; Kang and others, 2005). Therefore, in future experiments, it will be necessary to investigate whether astrogliosis is enhanced by transplanting NSC and to determine the effect of introducing exogenous astrocyte-like cells on the excitability of the brain. Indeed, one could imagine that an increase of the number of reactive astrocytes could lead to an aggravation of the epilepsy. If the latter would be the case it could be more advantageous to transplant alternative cell types which secrete seizure-suppressing substances but, in contrast to NSC, do not contribute to the neural cell compartment. For example, ex vivo modified fibroblasts could be used as these cells are able to stably survive for prolonged periods (up to one year) while continuously secreting a transgene protein (Blesch and others, 2001; Liu and others, 2002; Tobias and others, 2003; Pizzo and others, 2004).

On the other hand, introducing cells which directly integrate into the epileptic network may also be advantageous, especially if the donor-derived astrocytes are recruited instead of endogenous astrocytes. This would allow a more direct manipulation of the disease process and the epileptic network. For example, it has been demonstrated that the level of glutamine synthetase is decreased in an epileptic hippocampus (Eid and others, 2004). Glutamine synthetase is an enzyme which is expressed in astrocytes and is responsible for the metabolism of glutamate to glutamine. The defective breakdown of glutamate, which is an excitatory neurotransmitter, could be an important causative factor for the generation of seizures. Indeed, a microdialysis study in TLE patients has demonstrated that an epileptic seizure is preceded by increased levels of glutamate (During and Spencer, 1993). Therefore, genetic engineering of NSC prior to their transplantation to induce

overexpression of glutamine synthetase could be a strategy to reduce the extracellular levels of glutamate in the epileptic network and thereby reducing local excitability.

Together, NSC could be an interesting cell source for cell-based delivery purposes. However, further research is mandatory to identify conditions which can enhance the fraction of cells which survive after transplantation. It is also mandatory to find out whether the transplantation of NSC enhances the gliotic response and, if so, whether this aggravates the epileptic state.

CELL THERAPY FOR TLE THROUGH MODULATION OF ENDOGENOUS NEUROGENESIS

As already indicated in the introduction short and transient induction of global ischemia in the 4-VO model selectively affects hippocampal CA1 pyramidal neurons. In this model it was demonstrated that neural precursors residing in the posterior periventricular region (PPV) migrate towards the lesioned CA1 regions where they can replace CA1-like pyramidal cells (Nakatomi and others, 2002). Recently Parent and co-workers found that also in the PILO model for TLE precursor cells from the PPV migrate to the damaged hippocampal regions. However, in these hippocampal regions the precursor cells did not generate neurons, but exclusively differentiated towards glia. In contrast to the ischemically lesioned hippocampus there was no neural repair in this model for TLE (Parent and others, 2006b). Identification of the factors which promote neural replacement in case of an ischemic lesion and/or prevent regeneration in case of excitotoxic lesions could perhaps contribute to new strategies for stimulating endogenous repair in case of hippocampal sclerosis.

In the PILO model for TLE, but also in other TLE models, it was found that seizures enhance neurogenesis in the neurogenic regions: the olfactory system and the dentate gyrus (Parent and Lowenstein, 2002). In the SVZ of the PILO model for TLE, precursor cell proliferation was clearly upregulated after 2 hours of SE. BrdU labelling and focal injection of retroviral reporters into the rostral SVZ showed that most of the new neurons, generated in response to SE, migrate via the normal RMS pathway to their appropriate targets in the olfactory bulb. A small fraction of the SVZ-derived neuroblasts generated after SE left the RMS prematurely and migrated into injured forebrain regions. However, only a very small part of these new neurons persisted in these cortical regions for more than 5 weeks after SE. This indicates that even if neural replacement in cortical areas is present, it is only moderate (Parent and others, 2002). Studies investigating the possible role of this seizure-induced increase in SVZ neurogenesis have not been performed.

Most studies have focussed on seizure induced alterations in hippocampal neurogenesis, which is not surprising given the prominent role of the hippocampus in TLE. Those studies found that single seizures as well as prolonged SE severely influence granule cell neurogenesis in the dentate gyrus of the hippocampus. In contrast to most other disease models, seizure-induced neurogenesis is thought to contribute to the disease progression rather than being an endogenous repair mechanism.

Seizure activity has been demonstrated to stimulate proliferation of both radial glia-like NSC (Huttmann and others, 2003) and DCX positive migrating neuroblasts (Jessberger and others, 2005). This results in an increase

in net neurogenesis in the hippocampus of both kindling and SE models for TLE (Parent and others, 1997; Bengzon and others, 1997; Gray and Sundstrom, 1998; Parent and others, 1998; Scott and others, 1998; Parent, 2002; Jessberger and others, 2005; Smith and others, 2005; Overstreet-Wadiche and others, 2006). The majority of the newborn granule cells, generated in response to seizures, integrate appropriately into the GCL and develop the same morphological and functional properties as newborn granule neurons in non-epileptic rats. However, these developmental processes seem to be accelerated in response to seizures (Overstreet-Wadiche and others, 2006). The accelerated integration of higher number excitatory neurons could enhance excitability of hippocampal networks. In addition a portion of the newborn granule neurons show inappropriate migration, differentiation, and integration in response to seizures, further suggesting a pro-epileptic role of enhanced neurogenesis in response to seizures (Scharfman and others, 2000; Parent, 2002; Jessberger and others, 2005; Shapiro and Ribak, 2006; Parent and others, 2006a).

To further elaborate on the possible role of seizure-induced aberrant neurogenesis in epileptogenesis, we suppressed neurogenesis during hippocampal kindling acquisition, which is a validated model for temporal lobe epileptogenesis (Lothman and Williamson, 1994). To do this, the rat brain was exposed to low-dose (8Gy) radiation one day before the initiation of rapid kindling. This radiation dose was reported to selectively induce apoptosis in neuronal precursors and immature neurons, while leaving mature neurons and gliogenesis unaffected. By injecting rats with BrdU at the first and second kindling day and staining for the neuronal marker NeuN, three weeks after labelling, we found that neurogenesis was suppressed by more than 80% in the first days after radiation. Staining for DCX-positive neuroblasts at the end of the kindling acquisition, one week after radiation, showed that the number of neuroblasts at that time point was reduced by 95%. However, this suppression of neurogenesis did not prevent the rats from becoming fully kindled. Therefore, our results suggest that increased neurogenesis in response to seizures play no or only a limited role in kindling epileptogenesis and the establishment of hyperexcitable hippocampal networks.

In fact, we found that radiation-induced suppression of neurogenesis was associated with a more rapid kindling rate and more excitable hippocampal networks. Since it was reported that radiation could induce inflammation (Monje and others, 2003), we stained the rat brains at the end of the kindling acquisition for CD11 (activated microglia) and vimentin (activated astrocytes). We found significant inflammation in both radiated and non-radiated rats without differences in the extent between both groups. This indicates that radiation-induced inflammation is probably not responsible for the higher excitability observed in the radiated rats.

Based on these results it is tempting to speculate that seizure-induced neurogenesis enhances local inhibition. However, low-dose radiation is a rather unspecific tool which causes subtle side-effects such as changes in microvasculature and altered metabolic or signalling pathways (Silasi and others, 2004). Those side-effects, and not the suppression of neurogenesis, may be responsible for the higher excitability in response to radiation.

Taken together, our results indicate that enhanced neurogenesis does not contribute to the acquisition or the permanence of a fully kindled state and therefore does not play a major role in epileptogenesis. However, our

study does not allow discriminating between enhanced neurogenesis being an unspecific side-effect of seizure activity or endogenous protection mechanism against increased excitability. In a very recent study, Jakubs and colleagues compared seizure-generated granule neurons with granule neurons, generated in response to running. They found that new granule cells formed in context of seizures showed reduced excitability and demonstrated that this reduced excitability was the result of an increased inhibitory and decreased excitatory synaptic input on the developing new neurons (Jakubs and others, 2006). This shows that the addition of more excitatory neurons not necessarily results in an increase in net excitability. The authors hypothesized that the continuous addition of granule neurons with reduced excitatory and increased inhibitory synaptic input may have significant beneficial effects on the epileptic syndrome by increasing the threshold for seizure propagation through limbic circuitry.

In conclusion, although our study argues against a major role of seizure-induced neurogenesis in kindling epileptogenesis further studies in different TLE models and human patients are needed to further elaborate on the precise role of the seizure-generated neurons.

REFERENCES

- Abouelfetouh A, Kondoh T, Ehara K, Kohmura E. 2004. Morphological differentiation of bone marrow stromal cells into neuron-like cells after co-culture with hippocampal slice. *Brain Research* 1029(1):114-9.
- Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A. 2003. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 425(6961):968-73.
- Arida RM, Scorza FA, Peres CDA, Cavalheiro EA. 1999. The course of untreated seizures in the pilocarpine model of epilepsy. *Epilepsy Research* 34(2-3):99-107.
- Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ. 1998. Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats--similarities to astrocyte grafts. *Proc Natl Acad Sci U S A* 95(7):3908-13.
- Bengzon J, Kokaia Z, Elmer E, Nanobashvili A, Kokaia M, Lindvall O. 1997. Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. *Proc Natl Acad Sci U S A* 94(19):10432-7.
- Bertani N, Malatesta P, Volpi G, Sonogo P, Perris R. 2005. Neurogenic potential of human mesenchymal stem cells revisited: analysis by immunostaining, time-lapse video and microarray. *J Cell Sci* 118(Pt 17):3925-36.
- Bertram EH, Cornett JF. 1994. The evolution of a rat model of chronic spontaneous limbic seizures. *Brain Research* 661(1-2):157-62.
- Bittner RE, Schofer C, Weipoltshammer K, Ivanova S, Streubel B, Hauser E, Freilinger M, Hoger H, Elbe-Burger A, Wachtler F. 1999. Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. *Anat Embryol (Berl)* 199(5):391-6.
- Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlestedt C, Kim KS, Isacson O. 2002. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci U S A* 99(4):2344-9.
- Bjornson CR, Rietze RL, Reynolds BA, Magli MC, Vescovi AL. 1999. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* 283(5401):534-7.
- Black IB, Woodbury D. 2001. Adult rat and human bone marrow stromal stem cells differentiate into neurons. *Blood Cells Mol Dis* 27(3):632-6.
- Blesch A, Conner JM, Tuszynski MH. 2001. Modulation of neuronal survival and axonal growth in vivo by tetracycline-regulated neurotrophin expression. *Gene Ther* 8(12):954-60.
- Bragin A, Azizyan A, Almajano J, Wilson CL, Engel J, Jr. 2005. Analysis of chronic seizure onsets after intrahippocampal kainic acid injection in freely moving rats. *Epilepsia* 46(10):1592-8.
- Bragin A, Engel J, Jr., Wilson CL, Fried I, Mathern GW. 1999a. Hippocampal and entorhinal cortex high-frequency oscillations (100--500 Hz) in human epileptic brain and in kainic acid--treated rats with chronic seizures. *Epilepsia* 40(2):127-37.
- Bragin A, Engel J, Jr., Wilson CL, Vizentin E, Mathern GW. 1999b. Electrophysiologic analysis of a chronic seizure model after unilateral hippocampal KA injection. *Epilepsia* 40(9):1210-21.
- Bragin A, Wilson CL, Almajano J, Mody I, Engel J, Jr. 2004. High-frequency Oscillations after Status Epilepticus: Epileptogenesis and Seizure Genesis. *Epilepsia* 45(9):1017-23.
- Brazelton TR, Rossi FM, Keshet GI, Blau HM. 2000. From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* 290(5497):1775-9.
- Burns TC, Ortiz-Gonzalez XR, Gutierrez-Perez M, Keene CD, Sharda R, Demorest ZL, Jiang Y, Nelson-Holte M, Soriano M, Nakagawa Y, Luquin MR, Garcia-Verdugo JM, Prosper F, Low WC, Verfaillie CM. 2006. Thymidine Analogs Are Transferred from Prelabeled Donor to Host Cells in the Central Nervous System After Transplantation: A Word of Caution. *Stem Cells* 24(4):1121-7.
- Buzsaki G, Masliah E, Chen LS, Horvath Z, Terry R, Gage FH. 1991. Hippocampal grafts into the intact brain induce epileptic patterns. *Brain Res* 554(1-2):30-7.

- Buzsaki G, Ponomareff G, Bayardo F, Shaw T, Gage FH. 1988. Suppression and induction of epileptic activity by neuronal grafts. *Proc Natl Acad Sci U S A* 85(23):9327-30.
- Cavalheiro EA, Riche DA, Le Gal La Salle G. 1982. Long-term effects of intrahippocampal kainic acid injection in rats: a method for inducing spontaneous recurrent seizures. *Electroencephalogr Clin Neurophysiol* 53(6):581-9.
- Chen KA, Laywell ED, Marshall G, Walton N, Zheng T, Steindler DA. 2006. Fusion of neural stem cells in culture. *Experimental Neurology* 198(1):129-35.
- Chu K, Kim M, Jung KH, Jeon D, Lee ST, Kim J, Jeong SW, Kim SU, Lee SK, Shin HS, Roh JK. 2004. Human neural stem cell transplantation reduces spontaneous recurrent seizures following pilocarpine-induced status epilepticus in adult rats. *Brain Res* 1023(2):213-21.
- Clarke DL, Johansson CB, Wilbertz J, Veress B, Nilsson E, Karlstrom H, Lendahl U, Frisen J. 2000. Generalized potential of adult neural stem cells. *Science* 288(5471):1660-3.
- Clarke D, Frisen J. 2001. Differentiation potential of adult stem cells. *Current Opinion in Genetics & Development* 11(5):575-80.
- Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying QL, Cattaneo E, Smith A. 2005. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol* 3(9):e283.
- Conti L, Reitano E, Cattaneo E. 2006. Neural stem cell systems: diversities and properties after transplantation in animal models of diseases. *Brain Pathol* 16(2):143-54.
- Corti S, Locatelli F, Papadimitriou D, Donadoni C, Del Bo R, Fortunato F, Strazzer S, Salani S, Bresolin N, Comi GP. 2005. Multipotentiality, homing properties, and pyramidal neurogenesis of CNS-derived LeX(ssea-1) +/CXCR4+ stem cells. *FASEB J*:05-4170fje.
- D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. 2004. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci* 117(Pt 14):2971-81.
- Deng W, Obrocka M, Fischer I, Prockop DJ. 2001. In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. *Biochem Biophys Res Commun* 282(1):148-52.
- Dezawa M, Kanno H, Hoshino M, Cho H, Matsumoto N, Itokazu Y, Tajima N, Yamada H, Sawada H, Ishikawa H, Mimura T, Kitada M, Suzuki Y, Ide C. 2004. Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J Clin Invest* 113(12):1701-10.
- Dudek FE, Clark S, Williams PA, Grabenstatter HL. 2006. Kainate-induced status epilepticus: a chronic model of acquired epilepsy. In: Pitkanen A, Schwartzkroin PA, Moshé SL, editors. *Models of seizures and epilepsy*. Oxford: Elsevier Academic Press; p 415-32.
- During MJ, Spencer DD. 1993. Extracellular hippocampal glutamate and spontaneous seizure in the conscious human brain. *Lancet* 341(8861):1607-10.
- Eglitis MA, Mezey E. 1997. Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc Natl Acad Sci U S A* 94(8):4080-5.
- Eid T, Thomas MJ, Spencer DD, Runden-Pran E, Lai JC, Malthankar GV, Kim JH, Danbolt NC, Ottersen OP, de Lanerolle NC. 2004. Loss of glutamine synthetase in the human epileptogenic hippocampus: possible mechanism for raised extracellular glutamate in mesial temporal lobe epilepsy. *Lancet* 363(9402):28-37.
- Engel J. 1996. Introduction to temporal lobe epilepsy. *Epilepsy Research* 26(1):141-50.
- Englund U, Bjorklund A, Wictorin K, Lindvall O, Kokaia M. 2002. Grafted neural stem cells develop into functional pyramidal neurons and integrate into host cortical circuitry. *Proc Natl Acad Sci U S A* 99(26):17089-94.
- Evans MJ, Kaufman MH. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292(5819):154-6.
- Fedele DE, Koch P, Scheurer L, Simpson EM, Mohler H, Brustle O, Boison D. 2004. Engineering embryonic stem cell derived glia for adenosine delivery. *Neurosci Lett* 370(2-3):160-5.

- Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. 1998. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 279(5356):1528-30.
- French ED, Aldinio C, Schwarcz R. 1982. Intrahippocampal kainic acid, seizures and local neuronal degeneration: relationships assessed in unanesthetized rats. *Neuroscience* 7(10):2525-36.
- Furtado Mde A, Braga GK, Oliveira JA, Del Vecchio F, Garcia-Cairasco N. 2002. Behavioral, morphologic, and electroencephalographic evaluation of seizures induced by intrahippocampal microinjection of pilocarpine. *Epilepsia* 43(Suppl 5):37-9.
- Gage FH. 2000. Mammalian neural stem cells. *Science* 287(5457):1433-8.
- Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, Peterson DA, Suhr ST, Ray J. 1995. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci U S A* 92(25):11879-83.
- Galli R, Borello U, Gritti A, Minasi MG, Bjornson C, Coletta M, Mora M, De Angelis MG, Fiocco R, Cossu G, Vescovi AL. 2000. Skeletal myogenic potential of human and mouse neural stem cells. *Nat Neurosci* 3(10):986-91.
- Gernert M, Thompson KW, Loscher W, Tobin AJ. 2002. Genetically engineered GABA-producing cells demonstrate anticonvulsant effects and long-term transgene expression when transplanted into the central piriform cortex of rats. *Exp Neurol* 176(1):183-92.
- Glien M, Brandt C, Potschka H, Voigt H, Ebert U, Loscher W. 2001. Repeated low-dose treatment of rats with pilocarpine: low mortality but high proportion of rats developing epilepsy. *Epilepsy Research* 46(2):111-9.
- Gobbel GT, Choi SJ, Beier S, Niranjan A. 2003. Long-term cultivation of multipotential neural stem cells from adult rat subependyma. *Brain Res* 980(2):221-32.
- Goolsby J, Marty MC, Heletz D, Chiappelli J, Tashko G, Yarnell D, Fishman PS, Dhib-Jalbut S, Bever CT, Jr., Pessac B, Trisler D. 2003. Hematopoietic progenitors express neural genes. *Proc Natl Acad Sci U S A* 100(25):14926-31.
- Gorter JA, van Vliet EA, Aronica E, Lopes da Silva FH. 2001. Progression of spontaneous seizures after status epilepticus is associated with mossy fibre sprouting and extensive bilateral loss of hilar parvalbumin and somatostatin-immunoreactive neurons. *European Journal of Neuroscience* 13(4):657-69.
- Gray WP, Sundstrom LE. 1998. Kainic acid increases the proliferation of granule cell progenitors in the dentate gyrus of the adult rat. *Brain Res* 790(1-2):52-9.
- Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC. 1999. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* 401(6751):390-4.
- Guttinger M, Fedele D, Koch P, Padrun V, Pralong WF, Brustle O, Boison D. 2005a. Suppression of kindled seizures by paracrine adenosine release from stem cell-derived brain implants. *Epilepsia* 46(8):1162-9.
- Guttinger M, Padrun V, Pralong WF, Boison D. 2005b. Seizure suppression and lack of adenosine A1 receptor desensitization after focal long-term delivery of adenosine by encapsulated myoblasts. *Exp Neurol* 193(1):53-64.
- Harris RG, Herzog EL, Bruscia EM, Grove JE, Van Arnem JS, Krause DS. 2004. Lack of a Fusion Requirement for Development of Bone Marrow-Derived Epithelia. *Science* 305(5680):90-3.
- Hattiangady B, Rao MS, Zaman V, Shetty AK. 2006. Incorporation of embryonic CA3 cell grafts into the adult hippocampus at 4-months after injury: Effects of combined neurotrophic supplementation and caspase inhibition. *Neuroscience* 139(4):1369-83.
- Hellier JL, Patrylo PR, Buckmaster PS, Dudek FE. 1998. Recurrent spontaneous motor seizures after repeated low-dose systemic treatment with kainate: assessment of a rat model of temporal lobe epilepsy. *Epilepsy Res* 31(1):73-84.
- Hofstetter CP, Schwarz EJ, Hess D, Widenfalk J, El Manira A, Prockop DJ, Olson L. 2002. Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. *PNAS* 99(4):2199-204.
- Huber A, Padrun V, Deglon N, Aebischer P, Mohler H, Boison D. 2001. Grafts of adenosine-releasing cells suppress seizures in kindling epilepsy. *Proc Natl Acad Sci U S A* 98(13):7611-6.

- Hung SC, Cheng H, Pan CY, Tsai MJ, Kao LS, Ma HL. 2002. In vitro differentiation of size-sieved stem cells into electrically active neural cells. *Stem Cells* 20(6):522-9.
- Huttmann K, Sadgrove M, Wallraff A, Hinterkeuser S, Kirchhoff F, Steinhauser C, Gray WP. 2003. Seizures preferentially stimulate proliferation of radial glia-like astrocytes in the adult dentate gyrus: functional and immunocytochemical analysis. *European Journal of Neuroscience* 18(10):2769-78.
- Jackson KA, Mi T, Goodell MA. 1999. Hematopoietic potential of stem cells isolated from murine skeletal muscle. *Proc Natl Acad Sci U S A* 96(25):14482-6.
- Jakubs K, Nanobashvili A, Bonde S, Ekdahl CT, Kokaia Z, Kokaia M, Lindvall O. 2006. Environment Matters: Synaptic Properties of Neurons Born in the Epileptic Adult Brain Develop to Reduce Excitability. *Neuron* 52(6):1047-59.
- Jessberger S, Romer B, Babu H, Kempermann G. 2005. Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. *Exp Neurol* 196(2):342-51.
- Jessberger S, Clemenson GD, Jr., Gage FH. 2007. Spontaneous Fusion and Nonclonal Growth of Adult Neural Stem Cells. *Stem Cells* 25(4):871-4.
- Jiang Y, Henderson D, Blackstad M, Chen A, Miller RF, Verfaillie CM. 2003. Neuroectodermal differentiation from mouse multipotent adult progenitor cells. *Proc Natl Acad Sci U S A* 100 Suppl 1:11854-60.
- Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418(6893):41-9.
- Jin K, Mao XO, Batteur S, Sun Y, Greenberg DA. 2003. Induction of neuronal markers in bone marrow cells: differential effects of growth factors and patterns of intracellular expression. *Exp Neurol* 184(1):78-89.
- Kabos P, Ehtesham M, Kabosova A, Black KL, Yu JS. 2002. Generation of neural progenitor cells from whole adult bone marrow. *Exp Neurol* 178(2):288-93.
- Kang N, Xu J, Xu Q, Nedergaard M, Kang J. 2005. Astrocytic glutamate release-induced transient depolarization and epileptiform discharges in hippocampal CA1 pyramidal neurons. *J Neurophysiol* 94(6):4121-30.
- Keene CD, Ortiz-Gonzalez XR, Jiang Y, Largaespada DA, Verfaillie CM, Low WC. 2003. Neural differentiation and incorporation of bone marrow-derived multipotent adult progenitor cells after single cell transplantation into blastocyst stage mouse embryos. *Cell Transplant* 12(3):201-13.
- Kelly S, Bliss TM, Shah AK, Sun GH, Ma M, Foo WC, Masel J, Yenari MA, Weissman IL, Uchida N, Palmer T, Steinberg GK. 2004. Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. *PNAS* 101(32):11839-44.
- Kim BJ, Seo JH, Bubien JK, Oh YS. 2002. Differentiation of adult bone marrow stem cells into neuroprogenitor cells in vitro. *Neuroreport* 13(9):1185-8.
- Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, Gojo S, Taga T, Okano H, Hata J, Umezawa A. 2001. Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. *Differentiation* 68(4-5):235-44.
- Kopen GC, Prockop DJ, Phinney DG. 1999. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci U S A* 96(19):10711-6.
- Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M. 2000. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat Med* 6(11):1229-34.
- Leite JP, Babb TL, Pretorius JK, Kuhlman PA, Yeoman KM, Mathern GW. 1996. Neuron loss, mossy fiber sprouting, and interictal spikes after intrahippocampal kainate in developing rats. *Epilepsy Res* 26(1):219-31.
- Liu Y, Himes BT, Murray M, Tessler A, Fischer I. 2002. Grafts of BDNF-producing fibroblasts rescue axotomized rubrospinal neurons and prevent their atrophy. *Exp Neurol* 178(2):150-64.

- Lothman EW, Williamson JM. 1994. Closely spaced recurrent hippocampal seizures elicit two types of heightened epileptogenesis: a rapidly developing, transient kindling and a slowly developing, enduring kindling. *Brain Res* 649(1-2):71-84.
- Lowenstein DH, Seren MS, Longo FM. 1993. Prolonged increases in neurotrophic activity associated with kainate-induced hippocampal synaptic reorganization. *Neuroscience* 56(3):597-604.
- Lu P, Blesch A, Tuszynski MH. 2004. Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? *J Neurosci Res* 77(2):174-91.
- Lu P, Jones LL, Snyder EY, Tuszynski MH. 2003. Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. *Experimental Neurology* 181(2):115-29.
- Martin GR. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 78(12):7634-8.
- Martino G, Pluchino S. 2006. The therapeutic potential of neural stem cells. *Nat Rev Neurosci* 7(5):395-406.
- Matsui Y, Zsebo K, Hogan BLM. 1992. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* 70(5):841-7.
- Mazarati A, Bragin A, Baldwin R, Shin D, Wilson C, Sankar R, Naylor D, Engel J, Wasterlain CG. 2002. Epileptogenesis after self-sustaining status epilepticus. *Epilepsia* 43(Suppl 5):74-80.
- Mazarati AM, Wasterlain CG, Sankar R, Shin D. 1998. Self-sustaining status epilepticus after brief electrical stimulation of the perforant path. *Brain Research* 801(1-2):251-3.
- Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. 2000. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* 290(5497):1779-82.
- Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B. 2003. Transplanted bone marrow generates new neurons in human brains. *Proc Natl Acad Sci U S A* 100(3):1364-9.
- Monje ML, Toda H, Palmer TD. 2003. Inflammatory blockade restores adult hippocampal neurogenesis. *Science* 302(5651):1760-5.
- Munoz-Elias G, Marcus AJ, Coyne TM, Woodbury D, Black IB. 2004. Adult bone marrow stromal cells in the embryonic brain: Engraftment, migration, differentiation, and long-term survival. *J Neurosci* 24(19):4585-95.
- Muraoka K, Shingo T, Yasuhara T, Kameda M, Yuan W, Hayase H, Matsui T, Miyoshi Y, Date I. 2006. The high integration and differentiation potential of autologous neural stem cell transplantation compared with allogeneic transplantation in adult rat hippocampus. *Exp Neurol* 199(2):311-27.
- Nadler JV. 1981. Minireview. Kainic acid as a tool for the study of temporal lobe epilepsy. *Life Sci* 29(20):2031-42.
- Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M. 2002. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* 110(4):429-41.
- Neuhuber B, Gallo G, Howard L, Kostura L, Mackay A, Fischer I. 2004. Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. *J Neurosci Res* 77(2):192-204.
- Nissinen J, Halonen T, Koivisto E, Pitkanen A. 2000. A new model of chronic temporal lobe epilepsy induced by electrical stimulation of the amygdala in rat. *Epilepsy Res* 38(2-3):177-205.
- Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. 2001. Bone marrow cells regenerate infarcted myocardium. *Nature* 410(6829):701-5.
- Overstreet-Wadiche LS, Bromberg DA, Bensen AL, Westbrook GL. 2006. Seizures accelerate functional integration of adult-generated granule cells. *J Neurosci* 26(15):4095-103.
- Padovan CS, Jahn K, Birnbaum T, Reich P, Sostak P, Strupp M, Straube A. 2003. Expression of neuronal markers in differentiated marrow stromal cells and CD133+ stem-like cells 40. *Cell Transplant* 12(8):839-48.

- Parent JM. 2002. The role of seizure-induced neurogenesis in epileptogenesis and brain repair. *Epilepsy Res* 50(1-2):179-89.
- Parent JM, Elliott RC, Pleasure SJ, Barbaro NM, Lowenstein DH. 2006a. Aberrant seizure-induced neurogenesis in experimental temporal lobe epilepsy. *Ann Neurol* 59(1):81-91.
- Parent JM, Janumpalli S, McNamara JO, Lowenstein DH. 1998. Increased dentate granule cell neurogenesis following amygdala kindling in the adult rat. *Neurosci Lett* 247(1):9-12.
- Parent JM, Lowenstein DH. 2002. Seizure-induced neurogenesis: are more new neurons good for an adult brain? *Prog Brain Res* 135:121-31.
- Parent JM, Valentin VV, Lowenstein DH. 2002. Prolonged seizures increase proliferating neuroblasts in the adult rat subventricular zone-olfactory bulb pathway. *J Neurosci* 22(8):3174-88.
- Parent JM, von dem BN, Lowenstein DH. 2006b. Prolonged seizures recruit caudal subventricular zone glial progenitors into the injured hippocampus. *Hippocampus* 16(3):321-8.
- Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH. 1997. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci* 17(10):3727-38.
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science* 284(5411):143-7.
- Pizzo DP, Paban V, Coufal NG, Gage FH, Thal LJ. 2004. Long-term production of choline acetyltransferase in the CNS after transplantation of fibroblasts modified with a regulatable vector. *Brain Res Mol Brain Res* 126(1):1-13.
- Pluchino S, Zanotti L, Rossi B, Brambilla E, Ottoboni L, Salani G, Martinello M, Cattalini A, Bergami A, Furlan R, Comi G, Constantin G, Martino G. 2005. Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. *Nature* 436(7048):266-71.
- Priller J, Persons DA, Klett FF, Kempermann G, Kreutzberg GW, Dirnagl U. 2001. Neogenesis of cerebellar Purkinje neurons from gene-marked bone marrow cells in vivo. *J Cell Biol* 155(5):733-8.
- Qian L, Saltzman WM. 2004. Improving the expansion and neuronal differentiation of mesenchymal stem cells through culture surface modification. *Biomaterials* 25(7-8):1331-7.
- Quigg M, Straume M, Menaker M, Bertram EH. 1998. Temporal distribution of partial seizures: Comparison of an animal model with human partial epilepsy. *Annals of Neurology* 43(6):748-55.
- Ratajczak MZ, Kucia M, Reza R, Majka M, Janowska-Wieczorek A, Ratajczak J. 2004. Stem cell plasticity revisited: CXCR4-positive cells expressing mRNA for early muscle, liver and neural cells 'hide out' in the bone marrow
1. *Leukemia* 18(1):29-40.
- Resnick JL, Bixler LS, Cheng L, Donovan PJ. 1992. Long-term proliferation of mouse primordial germ cells in culture. *Nature* 359(6395):550-1.
- Reynolds BA, Weiss S. 1996. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* 175(1):1-13.
- Riban V, Boullieret V, Pham-Le BT, Fritschy J-M, Marescaux C, Depaulis A. 2002. Evolution of hippocampal epileptic activity during the development of hippocampal sclerosis in a mouse model of temporal lobe epilepsy. *Neuroscience* 112(1):101-11.
- Richardson RM, Fillmore HL, Holloway KL, Broaddus WC. 2004. Progress in cerebral transplantation of expanded neuronal stem cells. *J Neurosurg* 100(4):659-71.
- Richardson RM, Broaddus WC, Holloway KL, Sun D, Bullock MR, Fillmore HL. 2005. Heterotypic neuronal differentiation of adult subependymal zone neuronal progenitor cells transplanted to the adult hippocampus. *Molecular and Cellular Neuroscience* 28(4):674-82.
- Ruschenschmidt T, Koch PG, Brustle O, Beck H. 2005. Functional properties of ES cell-derived neurons engrafted into the hippocampus of adult normal and chronically epileptic rats. *Epilepsia* 46:174-83.

- Ryu JK, Kim J, Cho SJ, Hatori K, Nagai A, Choi HB, Lee MC, McLarnon JG, Kim SU. 2004. Proactive transplantation of human neural stem cells prevents degeneration of striatal neurons in a rat model of Huntington disease. *Neurobiology of Disease* 16(1):68-77.
- Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. 2000. Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp Neurol* 164(2):247-56.
- Scharfman HE, Goodman JH, Sollas AL. 2000. Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. *J Neurosci* 20(16):6144-58.
- Schwarcz R, Zaczek R, Coyle JT. 1978. Microinjection of kainic acid into the rat hippocampus. *Eur J Pharmacol* 50(3):209-20.
- Scott BW, Wang S, Burnham WM, De Boni U, Wojtowicz JM. 1998. Kindling-induced neurogenesis in the dentate gyrus of the rat. *Neurosci Lett* 248(2):73-6.
- Shamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, Blumenthal PD, Huggins GR, Gearhart JD. 1998. Derivation of pluripotent stem cells from cultured human primordial germ cells. *PNAS* 95(23):13726-31.
- Shapiro LA, Ribak CE. 2006. Newly born dentate granule neurons after pilocarpine-induced epilepsy have hilar basal dendrites with immature synapses. *Epilepsy Res* 69(1):53-66.
- Shetty AK, Turner DA. 1997. Development of long-distance efferent projections from fetal hippocampal grafts depends upon pathway specificity and graft location in kainate-lesioned adult hippocampus. *Neuroscience* 76(4):1205-19.
- Shetty AK, Zaman V, Turner DA. 2000. Pattern of long-distance projections from fetal hippocampal field CA3 and CA1 cell grafts in lesioned CA3 of adult hippocampus follows intrinsic character of respective donor cells. *Neuroscience* 99(2):243-55.
- Shihabuddin LS, Hertz JA, Holets VR, Whittemore SR. 1995. The adult CNS retains the potential to direct region-specific differentiation of a transplanted neuronal precursor cell line. *J Neurosci* 15(10):6666-78.
- Shihabuddin LS, Holets VR, Whittemore SR. 1996. Selective hippocampal lesions differentially affect the phenotypic fate of transplanted neuronal precursor cells. *Exp Neurol* 139(1):61-72.
- Shihabuddin LS, Horner PJ, Ray J, Gage FH. 2000. Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. *J Neurosci* 20(23):8727-35.
- Silasi G, az-Heijtz R, Besplug J, Rodriguez-Juarez R, Titov V, Kolb B, Kovalchuk O. 2004. Selective brain responses to acute and chronic low-dose X-ray irradiation in males and females. *Biochemical and Biophysical Research Communications* 325(4):1223-35.
- Smith PD, Mclean KJ, Murphy MA, Turnley AM, Cook MJ. 2005. Seizures, not hippocampal neuronal death, provoke neurogenesis in a mouse rapid electrical amygdala kindling model of seizures. *Neuroscience* 136(2):405-15.
- Stafstrom CE, Thompson JL, Holmes GL. 1992. Kainic acid seizures in the developing brain: status epilepticus and spontaneous recurrent seizures. *Brain Res Dev Brain Res* 65(2):227-36.
- Suhonen JO, Peterson DA, Ray J, Gage FH. 1996. Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. *Nature* 383(6601):624-7.
- Sun YE, Martinowich K, Ge W. 2003. Making and repairing the mammalian brain--signaling toward neurogenesis and gliogenesis. *Semin Cell Dev Biol* 14(3):161-8.
- Svendsen CN, Caldwell MA, Shen J, ter Borg MG, Rosser AE, Tyers P, Karmiol S, Dunnett SB. 1997. Long-Term Survival of Human Central Nervous System Progenitor Cells Transplanted into a Rat Model of Parkinson's Disease. *Experimental Neurology* 148(1):135-46.

- Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW. 2002. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* 416(6880):542-5.
- Thompson K, Anantharam V, Behrstock S, Bongarzone E, Campagnoni A, Tobin AJ. 2000. Conditionally immortalized cell lines, engineered to produce and release GABA, modulate the development of behavioral seizures. *Exp Neurol* 161(2):481-9.
- Thompson KW. 2005. Genetically engineered cells with regulatable GABA production can affect afterdischarges and behavioral seizures after transplantation into the dentate gyrus. *Neuroscience* 133(4):1029-37.
- Thompson KW, Suchomelova LM. 2004. Transplants of cells engineered to produce GABA suppress spontaneous seizures. *Epilepsia* 45(1):4-12.
- Tian GF, Azmi H, Takano T, Xu Q, Peng W, Lin J, Oberheim N, Lou N, Wang X, Zielke HR, Kang J, Nedergaard M. 2005. An astrocytic basis of epilepsy. *Nat Med* 11(9):973-81.
- Tobias CA, Shumsky JS, Shibata M, Tuszyński MH, Fischer I, Tessler A, Murray M. 2003. Delayed grafting of BDNF and NT-3 producing fibroblasts into the injured spinal cord stimulates sprouting, partially rescues axotomized red nucleus neurons from loss and atrophy, and provides limited regeneration. *Exp Neurol* 184(1):97-113.
- Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, Miller FD. 2001. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 3(9):778-84.
- Tondreau T, Lagneaux L, Dejeneffe M, Massy M, Mortier C, Delforge A, Bron D. 2004. Bone marrow-derived mesenchymal stem cells already express specific neural proteins before any differentiation. *Differentiation* 72(7):319-26.
- Tsai RY, McKay RD. 2000. Cell contact regulates fate choice by cortical stem cells. *J Neurosci* 20(10):3725-35.
- Turner DA, Shetty AK. 2003. Clinical prospects for neural grafting therapy for hippocampal lesions and epilepsy. *Neurosurgery* 52(3):632-44.
- Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M. 2003. Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* 422(6934):897-901.
- Watt FM, Hogan AB. 2000. Out of Eden: Stem Cells and Their Niches. *Science* 287(5457):1427-30.
- Wernig M, Benninger F, Schmandt T, Rade M, Tucker KL, Bussow H, Beck H, Brustle O. 2004. Functional integration of embryonic stem cell-derived neurons in vivo. *J Neurosci* 24(22):5258-68.
- Wieser HG. 2004. Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis. *Epilepsia* 45(6):695-714.
- Winkler C, Fricker RA, Gates MA, Olsson M, Hammang JP, Carpenter MK, Bjorklund A. 1998. Incorporation and glial differentiation of mouse EGF-responsive neural progenitor cells after transplantation into the embryonic rat brain. *Mol Cell Neurosci* 11(3):99-116.
- Wislet-Gendebien S, Hans G, Leprince P, Rigo JM, Moonen G, Rogister B. 2005. Plasticity of Cultured Mesenchymal Stem Cells: Switch from Nestin-Positive to Excitable Neuron-Like Phenotype. *Stem Cells* 23(3):392-402.
- Woodbury D, Reynolds K, Black IB. 2002. Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. *J Neurosci Res* 69(6):908-17.
- Woodbury D, Schwarz EJ, Prockop DJ, Black IB. 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 61(4):364-70.
- Wurmser AE, Nakashima K, Summers RG, Toni N, D'Amour KA, Lie DC, Gage FH. 2004. Cell fusion-independent differentiation of neural stem cells to the endothelial lineage. *Nature* 430(6997):350-6.
- Yan J, Welsh AM, Bora SH, Snyder EY, Koliatsos VE. 2004. Differentiation and tropic/trophic effects of exogenous neural precursors in the adult spinal cord. *J Comp Neurol* 480(1):101-14.
- Ying QL, Nichols J, Evans EP, Smith AG. 2002. Changing potency by spontaneous fusion. *Nature* 416(6880):545-8.

Yoon Ys, Wecker A, Heyd L, Park JS, Tkebuchava T, Kusano K, Hanley A, Scadova H, Qin G, Cha DH, Johnson KL, Aikawa R, Asahara T, Losordo DW. 2005. Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. *J Clin Invest* 115(2):326-38.

Zaman V, Shetty AK. 2002. Combined neurotrophic supplementation and caspase inhibition enhances survival of fetal hippocampal CA3 cell grafts in lesioned CA3 region of the aging hippocampus. *Neuroscience* 109(3):537-53.

Zaman V, Shetty AK. 2003. Fetal hippocampal CA3 cell grafts enriched with fibroblast growth factor-2 exhibit enhanced neuronal integration into the lesioned aging rat hippocampus in a kainate model of temporal lobe epilepsy. *Hippocampus* 13(5):618-32.

Zaman V, Turner DA, Shetty AK. 2000. Survival of grafted fetal neural cells in kainic acid lesioned CA3 region of adult hippocampus depends upon cell specificity. *Exp Neurol* 161(2):535-61.

Zaman V, Turner DA, Shetty AK. 2001. Prolonged postlesion transplantation delay adversely influences survival of both homotopic and heterotopic fetal hippocampal cell grafts in Kainate-lesioned CA3 region of adult hippocampus. *Cell Transplant* 10(1):41-52.

Chapter 8

General Conclusion

This chapter provides a general conclusion with regard to the research questions formulated in the introduction and clarifies how our results contribute to a further understanding of the feasibility of cell therapy approaches as potential treatment for TLE. In some answers specific considerations about the used methodology are presented.

CELL TRANSPLANTATION FOR TEMPORAL LOBE EPILEPSY:

A. Neuropotency of multipotent adult progenitor-like cells and adult neural stem cells

- 1. Can we isolate cell clones from the bone marrow which display the high proliferative potential, morphology and phenotypic profile as described for multipotent adult progenitor cells (MAPC)?**

Using the protocol which was originally described for the isolation and culture of MAPC and subsequent single cell cloning, we were able to generate cell clones with a high proliferative potential (more than 40 passages) and a spindle- to triangle-shaped morphology. Among those, we could identify clones which were negative for MHC I and MHC II and did not or only very moderately express CD44.

- 2. Are the MAPC-like cells pluripotent? In other words can we differentiate them towards endodermal (endothelium), mesodermal (hepatocytes) and ectodermal (neuronal) cells?**

Despite a high number of attempts we were not able to reproducibly and inarguably differentiate the MAPC-like cells towards endothelial cells, hepatocytes or neuronal cells by using the protocols described for MAPC differentiation. RT-PCR, flow cytometry and immunohistochemistry analysis showed that expression of differentiation markers was incongruent and incomplete.

- 3. Can we isolate neurosphere-forming neural stem cell (NSC) clones from the SVZ of adult rodent brain?**

By plating SVZ-derived cells in a chemically-defined expansion medium after mechanical and enzymatic dissociation of the microdissected SVZ tissue we obtained neurosphere cultures. These neurospheres could be mechanically dissociated and further expanded for an extended number of passages.

- 4. Can these NSC be differentiated towards the three neural lineages: neurons, oligodendrocytes and astrocytes?**

From these neurosphere cultures single cell-derived clones could be produced which were able to generate the three neural lineages: neurons, astrocytes and oligodendrocytes.

- 5. Which *in vitro* differentiation protocols can be used to compare neuropotency of both MAPC-like cells and NSC?**

Four protocols were selected which had been reported to induce neural transdifferentiation of bone marrow-derived cells: the Jiang method, the Kohyama method, the Kabos method, the Woodbury

method. The Woodbury method was discarded because it caused massive cell death of the NSC. Using the other three methods the NSC acquired complex neuronal and glial morphologies. The MAPC-like cells only morphologically resembled (immature) neurons using the Jiang method.

6. Which techniques can be used to evaluate neuropotency?

Next to analysing morphology of the differentiated cells, we used real-time PCR and immunocytochemistry to screen the expression of neuroglial genes and antigens respectively. Both techniques showed upregulation or *de novo* expression of all investigated neuroglial markers in NSC, thereby confirming the ability of the three remaining protocols to induce neural differentiation of neuropotent cell types. Our results do however indicate that analysis of morphology and screening of marker expression to proof neural transdifferentiation should be evaluated very carefully.

7. Is there a difference in the neuropotency of MAPC-like cells and NSC?

MAPC-like cells adopted only in one protocol neural morphologies and expressed only two of the six evaluated neural markers, without upregulation of their expression after differentiation. We therefore assessed that neural morphology and expression of both antigens were aspecific rather than a proof of neuropotency. Neural differentiation of NSC was much more robust and reproducible which indicates that neuropotency of NSC was higher than that of MAPC-like cells. Based on these results NSC were chosen as cell source for future transplantation.

Consideration about the methodology: Adoption of complex neuronal and glial morphologies and upregulation of all investigated neuronal and glial marker genes by the NSC in three different differentiation protocol is still no absolute proof for the ability to generate functional neuronal or glial cells. To proof functionality electrophysiological studies (patch clamp, calcium imaging) need to be done.

B. Intrahippocampal kainic acid model for temporal lobe epilepsy

1. What is the mortality rate after SE induced by intrahippocampal KA injection?

Intrahippocampal KA injection resulted in SE in all 15 treated rats. All rats survived although in two rats SE was interrupted by injecting pentobarbital because they displayed extremely wild running and jumping behaviour.

2. What is the fraction of rats displaying spontaneous seizures after intrahippocampal KA injection?

Spontaneous seizures were detected in all 15 rats. In the 2 of the 15 rats seizures were only detected in the second monitoring period, 40 to 42 weeks after SE.

Consideration about the methodology: Since the capacity of our video-EEG setup only allowed the recording of video-EEG for 72 consecutive hours/week during the first monitoring period rare seizures could be missed leading to an underestimation of the fraction of rats displaying spontaneous seizures.

3. At what time following KA-induced SE can spontaneous seizures be detected?

In 13 of the 15 rats at least one seizure was detected in the first eight weeks after SE. The mean latency between the SE and the first detected seizures in these 13 rats was about 28 days.

Consideration about the methodology: As we only record for 72 consecutive hours per week during the first monitoring period (see point 2) the latent period (time between SE and first seizure) could not be determined.

4. What is the behavioural component of the spontaneous seizures?

Two types of clinical seizures could be discriminated. The majority of the seizures (66%) were partial seizures with secondary generalization (SG). The remaining seizures did not secondarily generalize and no convulsive behaviour could be detected.

5. What is the mean duration of the spontaneous seizures?

The spontaneous seizures lasted in average ± 64 s. Partial seizures with SG had a significantly longer duration (± 72 s) than partial seizures that did not secondarily generalize (± 46 s).

6. Is there a large variation in seizure frequency among rats?

Mean seizure frequency varied considerably among rats (0 to 7.4 seizures per day). In general, seizure frequency was relatively low (± 0.56 seizures per day) with 11 of the 15 rats displaying less than one spontaneous seizure per day.

7. Is there a progression in seizure frequency?

In general, we observed a progression in seizure frequency. In the four rats with the highest number of seizures a significant correlation between time and seizure frequency could be demonstrated.

8. Do the spontaneous seizures occur in a circadian rhythm?

In the rats which had more than one seizure per day, more seizures occurred during daytime (lights on).

9. Could the intrahippocampal KA model be used for the evaluation of anti-epileptic effects of cell transplantation?

Because the frequency of spontaneous seizures in the intrahippocampal KA model is rather low and highly variable, a lot of animals will be needed to screen anti-epileptic effects of cell transplantation or

other treatments. However, this model is very interesting to evaluate the effect of cell transplantation in the sclerotic hippocampus since the extent of the initial KA-induced damage is limited and most seizures originate from the lesioned hippocampus.

C. Transplantation of adult neural stem cells in the intrahippocampal kainic acid model

1. Do NSC survive after transplantation in the intact or KA-lesioned hippocampus?

Both in the intact and the sclerotic hippocampus a small fraction of approximately 1% of the 140,000 GFP/BrdU double-labelled cells were detected three and six weeks after transplantation.

Consideration about the methodology: Many cells were detected which were only labelled with BrdU and not with GFP. Since it cannot be excluded that BrdU was transferred from dying transplanted cells to endogenous dividing precursors we only counted GFP/BrdU double-labelled cells. This could be an underestimation of the survival if it turns out that the effect is due to a downregulation of GFP.

2. Is the survival rate affected due to the presence of the lesion?

The number of donor-derived cells in intact and sclerotic hippocampus was not significantly different which indicates that the survival rate of the transplanted cells was not affected by the presence of the KA lesion.

3. Are the dispersion and/or migration of the transplanted cells different in intact compared to lesioned hippocampus?

In the intact hippocampus most of the transplanted cells aligned the fracture planes of the hippocampus. A small fraction of the transplanted cells had migrated towards the granule cell layer (GCL). In the sclerotic hippocampus the transplanted cells remained in dense clusters. No donor-derived cells were detected in the GCL.

4. To which type of cells do NSC differentiate after transplantation?

Both in the intact and sclerotic hippocampus transplanted NSC differentiated mainly towards astrocytes and only a small fraction differentiated towards neurons. In the intact hippocampus NSC-derived neurons were exclusively detected in the GCL. In the sclerotic hippocampus donor-derived neurons were found within the cluster of transplanted cells.

5. Is the cell fate of the NSC influenced by the presence of the lesion?

The presence of the KA lesion had no major effect on neuronal differentiation but clearly stimulated differentiation towards astrocytes. This indicates that the transplanted NSC contributed to the gliotic response on the KA lesion.

6. Are outcome parameters of the transplantation (survival, dispersion, integration and differentiation) affected by a longer delay between the lesion and the transplantation?

Survival of the transplanted cells was significantly lower if the delay between the lesion and the transplantation was longer (three weeks compared to three days). A longer postlesion delay had no major influence on the dispersion or the differentiation of the cells.

7. Is the survival of NSC stable over time?

The number of transplanted cells detected three and six weeks after transplantation did not significantly differ. So even though the fraction of surviving cells was low, survival of the transplanted cells was relatively stable over time.

8. Could NSC be used for the structural repair of damaged epileptic networks in the intrahippocampal kainic acid model?

As stated higher, only a small fraction of the transplanted NSC differentiated towards neurons upon transplantation in the sclerotic hippocampus. Those donor-derived neurons remained within the transplanted cell cluster. Taken together, there was little proof for neuronal replacement or circuitry repair in the sclerotic hippocampus of the intrahippocampal KA model.

9. Could NSC be used for the local delivery of seizure-modifying substances?

Based on their relative stable survival, NSC could be used for local delivery approaches. NSC could be engineered prior to their transplantation in order to induce them to secrete seizure suppressing substances. However, further research is necessary to increase the fraction of NSC that survives after transplantation.

1. Can low-dose, whole brain radiation suppress neurogenesis in the rapid kindling model for TLE?

Exposure of the whole rat brain to 8 Gy radiation, one day before the initiation of rapid hippocampal kindling, suppressed seizure-induced granule cell neurogenesis during kindling acquisition. In the first three days after radiation the number of newborn neurons was 83% less in radiated compared to non-radiated rats. At the end of the kindling acquisition, one week after radiation and after 48 kindling stimulations, the number of neuroblasts in the hippocampus was 95% less in radiated compared to non-radiated rats.

2. Is there a permanent suppression of neurogenesis?

Three weeks after radiation and after 70 kindling stimulations there was a partial recovery of neuroblast production. At that time point, the number of neuroblasts in the hippocampus of radiated rats was 56 % less than in non-radiated rats.

3. Is there a difference in afterdischarge threshold (ADT) and afterdischarge duration (ADD) between radiated and non-radiated rats?

In non-radiated rats the ADT increased during hippocampal kindling while it did not in radiated rats. As a result the threshold to evoke a seizure became significantly lower in radiated rats.

No difference in ADD between radiated and non-radiated rats was found throughout kindling.

4. Does radiation affect kindling rate?

At the end of the kindling acquisition, five to seven days after radiation, kindling rate was enhanced in radiated rats. Both the lower ADT and the higher kindling rate reflected a higher excitability in radiated rats.

5. Is brain inflammation different between radiated and non-radiated rats?

Both in radiated and non-radiated rats there was evidence of brain inflammation. However, quantitative analysis could not demonstrate differences in the extent of inflammation. This indicates that inflammation was probably not the main mechanism behind radiation-induced increase in excitability.

Consideration about the methodology: Besides inflammation radiation can cause other, more subtle, side-effects such as changes in microvasculature or alterations in metabolic or signalling pathways. Those side-effects could also be responsible for the enhanced excitability during kindling. So, a causative link between reduced neurogenesis and enhanced excitability cannot be made based on our experiment.

6. Can radiated rats become permanently fully kindled?

Despite the significant suppression of seizure-induced neurogenesis radiated rats became fully kindled and displayed a tonic-clonic seizure in response to each kindling stimulation. As stated higher, radiation even enhanced the kindling rate at the end of kindling acquisition.

7. Does radiation affect permanence of the fully kindled state?

Radiated rats still displayed tonic-clonic seizures in response to kindling during the retest phase. Therefore, seizure-induced neurogenesis does not seem to play a major role in the establishment of a permanent hyperexcitable fully kindled state.

8. Could suppression of seizure-induced neurogenesis be a potential cell therapy for TLE?

Our results indicate that suppression of seizure-induced neurogenesis did not slow down or prevent epileptogenesis. So, blocking neurogenesis has little chance to become a possible treatment for TLE. In fact, epileptogenesis was enhanced after radiation. This could be the result of an aspecific side effect of radiation or it could indicate that enhanced neurogenesis in response to seizures is an attempt to lower hyperexcitability. If the latter would be the case, it may be interesting to investigate the effect of stimulating seizure-induced neurogenesis.

Chapter 9

Future Perspectives

Reconstruction of damaged neuronal circuitry and subsequent restoration of normal brain function is the ultimate goal of cell transplantation for neurodegenerative diseases. Although neuropotent stem cells are believed to be ideal candidate donor cells, studies showing functional replacement of lost neurons by transplanting neuropotent stem cells have been extremely limited. Stem cells which can be differentiated towards neurons *in vitro* only rarely generate significant numbers of neurons upon transplantation in the damaged brain. This indicates that substantial efforts are required to identify factors which mediate the permissiveness of the environment to support neuronal differentiation and repair. The most promising study so far was done by Kim and coworkers. In this study ESC were predifferentiated *in vitro* towards highly enriched midbrain NSC using a complex and multi-step protocol. Upon transplantation into a rat model for PD, these midbrain NSC generated high amounts of dopaminergic neurons which extended axons to the striatum, formed functional synaptic connections and induced subsequent behavioural recovery (Kim and others, 2002). This report points out that unravelling neuronal differentiation pathways and generation specific neuronal precursor phenotypes is of crucial importance.

In case of TLE with HS, neuronal damage and circuitry that needs to be restored is much more complex than in PD. Functional cell replacement in TLE will require the *in vitro* production of different precursor cell populations of CA1 and CA3 pyramidal neurons but also of different types of interneurons and hilar mossy cells. The sclerotic environment will need to be modulated in such a way that the grafted cells can migrate to appropriate cell layers and reconnect in a proper way in order to restore the balance between excitation and inhibition and the functionality of hippocampal networks. To fulfil these requirements, a lot of additional research is needed to further unravel the signalling pathways and pattern determination involved in hippocampal development and hippocampal sclerosis.

Several studies have shown that behavioural recovery after NSC transplantation in a variety of neurodegenerative disease models is the result of trophic or neuroprotective effects of NSC rather than contribution of the transplanted NSC to functional reconstruction of the damaged circuitry (Lu and others, 2003; Yan and others, 2004; Ryu and others, 2004; Pluchino and others, 2005). In the PILO model for chronic TLE, systemic injection of NSC one day after SE resulted in the incorporation of NSC-derived cells in different damaged limbic brain areas. Although there was no functional repair of the damaged circuitry, clear anti-epileptogenic effects of the transplantation were reported (Chu and others, 2004). This could be the result of growth factor secretion by the transplanted NSC and subsequent survival of limbic neurons which normally die after SE. If so, strategies in which NSC are used for the secretion of trophic factors or neuroprotective molecules could further enhance the anti-epileptogenic effect. In this context NSC have been engineered in order to secrete high numbers of neuroprotective molecules such as GDNF (Behrstock and others, 2005; Klein and others, 2005), NGF (Andsberg and others, 1998) and NT-3 (Liu and others, 1999) and transplanted in disease models such as PD, ALS and stroke. As a result of transplantation, protection of endogenous neurons was demonstrated.

Sofar, transplantation of cells for the local delivery of anti-seizure substances has proved to be the easiest and most promising cell therapy strategy for TLE. In this dissertation, we already discussed the different cell-based delivery approaches, using different fetal and non-fetal cell types, with sometimes very potent seizure suppressing effects. In most cases the anti-seizure effects were only transient due to the progressive death of the transplanted cells. Another major limitation of most transplantation studies was that cells were transplanted prior to epileptogenesis in models which do not display hippocampal sclerosis or spontaneous seizures. Therefore it is difficult to extrapolate the results to human TLE. In our study, we showed that exogenous adult-derived NSC are able to stably survive in a sclerotic hippocampus of a TLE model with spontaneous seizures. Therefore, NSC are a promising donor cell type to be investigated for future use in cell-based delivery strategies for TLE.

Most successful results in the context of cell-based delivery of seizure-suppressing substances have been obtained with encapsulated adenosine-releasing cells transplanted into the lateral ventricle of fully kindled rats. For as long as the cells survived, a complete suppression of seizures was seen. The adenosine releasing cells were obtained by disruption of the gene encoding for the adenosine metabolizing enzyme, adenosine kinase, through chemical mutagenesis (fibroblasts or myoblasts) or genetic engineering (ESC) (Huber and others, 2001; Fedele and others, 2004; Guttinger and others, 2005a; Guttinger and others, 2005b). Genetic engineering of ESC has led to the production of a transgenic $Adk^{+/-}$ mouse line. By crossing these $Adk^{+/-}$ mice homozygous $Adk^{-/-}$ mice can be generated, which only survive for about 14 days after their birth (Boison and others, 2002). In collaboration with Dr. Detlev Boison, from the RS Dow Neurobiology Laboratories (Portland, Oregon, USA), we will try to isolate NSC from the brain of early postnatal $Adk^{-/-}$ mice. Based on the anticonvulsive effects in the kindling model, transplantation of adenosine-releasing NSC, derived from $Adk^{-/-}$ mice, may result in anti-epileptogenic and/or antiepileptic effects after transplantation in the sclerotic hippocampus of the intrahippocampal KA model.

Next to adenosine, other neuromodulators such as different neuropeptides (galanin, somatostatin, neuropeptide Y and dynorphin) exert seizure-suppressing effects and are believed to be physiologically active as endogenous anticonvulsants (Zini and others, 1993; Greber and others, 1994; Qian and others, 1997; Terman and others, 2000). These neuropeptides could be very interesting candidate molecules for cell based delivery strategies. In fact endogenous neurons have been transfected using a viral vector, carrying the gene for galanin and neuropeptide Y, to induce secretion of the neuropeptides by the neurons (*in vivo* gene therapy).

Virus-mediated induction of galanin secretion in endogenous neurons of the hippocampus (Haberman and others, 2003; Lin and others, 2003), the inferior collicular cortex (Haberman and others, 2003) and the pyriform cortex (McCown, 2006) resulted in an increased threshold to evoke seizures by electrical stimulation (Haberman and others, 2003; McCown, 2006) and a decrease or even complete suppression of seizures induced by respectively intrahippocampal (Lin and others, 2003) or intraperitoneal injection of kainic acid (McCown, 2006).

Using a similar approach hippocampal cells have been induced to secrete neuropeptide Y (NP-Y). This manipulation prevented the development of SE in response to intrahippocampal KA injection, retarded the kindling acquisition and increased the threshold to electrically evoke a seizure (Richichi and others, 2004).

In these studies, *in vivo* gene therapy always preceded the induction of the epileptic state and the effect on spontaneous seizures was not investigated. Spontaneous seizure models are characterized by extensive and progressive neuronal cell death, so the use of endogenous neurons for secreting of the neuropeptide may be a limiting factor. Therefore, strategies in which NSC are genetically engineered to release those seizure-suppressing neuropeptides, prior to transplantation, may be more optimal. Another advantage of this ex-vivo gene therapy strategy is that cells can be enriched and characterized before transplantation which may augment reproducibility.

In our transplantation study, the fraction of adult NSC that survived transplantation was rather low, compared to other studies, even in the intact hippocampus. Consequently, high numbers of cells will need to be injected to yield sufficient release of antiepileptic factors. Therefore, modifications that could contribute to enhanced survival will be tested in the future. This includes deriving NSC from other sources (embryonic brain, adult hippocampus), the use of other culture methods (monolayer cultures), pre-treating the cells with growth factors and anti-apoptotic substances, performing autologous transplantation and/or more effective immunosuppression strategies (combination of different immunosuppressants).

Although we have demonstrated that a fraction of the transplanted NSC is able to survive for at least six weeks after transplantation, it could be that prolonged survival of transplanted NSC is compromised by genetic engineering and/or the continuous secretion of seizure suppressants. Therefore, it is mandatory to evaluate long-term survival of *ex vivo* manipulated NSC upon transplantation. If manipulated cells are unable to survive for extended periods, continuous infusion with growth factors and/or anti-apoptotic substances could be evaluated as a strategy to promote long-term survival.

Even if cells survive for prolonged periods, seizure suppression could be only temporary due to suppression of the secretion of the transgene by the donor cells or downregulation of receptor expression by the host cells. Therefore it will need to be confirmed whether possible seizure-suppressing effects of transplantation are stable and long lasting.

In the light of possible clinical applications of NSC transplantation, it will be necessary to obtain NSC from human sources. One possible source could be human ESC. However further work needs to be done to safely and adequately derive phenotypically restricted neuronal and oligodendrocytic progenitors to eliminate the possibility that implantation of undifferentiated ESC generate teratocarcinomas. Another possibility is to derive NSC from human embryonic (Vescovi and others, 1999; Svendsen and Caldwell, 2000) or adult primary brain tissue (Akiyama and others, 2001; Westerlund and others, 2005). Human-derived NSC can be genetically engineered to secrete specific proteins. For example human forebrain-derived NSC have been genetically engineered to release GDNF and transplanted into the brain of animal models for ALS and PD. In both cases the

transplanted cells survived and continued to secrete GDNF (Behrstock and others, 2005; Klein and others, 2005). In analogy, human NSC could be engineered to deliver seizure-suppressing substances prior to their transplantation into animal models with spontaneous seizures.

Eventually, *ex vivo* manipulated human NSC, secreting seizure-suppressing substances, could be used for transplantation in human refractory epilepsy patients in whom the seizure focus or foci are located in eloquent cortex. However, before going to a clinical context it will be necessary to assess the safety of transplanting genetically engineered cells. For example, genetic engineering may induce additional mutations which could render a cell line tumorigenic. Moreover, uncontrolled release of the transgene could have detrimental effects. Therefore transgene expression will need to be controlled. To date, regulatable promoter systems are already available, e.g. doxycycline inducible or repressible promoters. However, a more subtle regulation of transgene expression than a simple “on”-“off” situation might be required.

MODULATION OF ENDOGENOUS NEUROGENESIS IN CASE OF TLE

The role of enhanced dentate granule cell neurogenesis in response to seizures remains unclear. As stated earlier, a part of the newborn neurons contribute to the formation of abnormal circuitry by their migration to ectopic locations and/or the formation of recurrent synaptic connections (Scharfman and others, 2000; Parent and Lowenstein, 2002; Shapiro and others, 2005). The great majority of the newborn neurons integrates normally in the GCL but their maturation and synaptic integration is accelerated upon seizure-activity (Overstreet-Wadiche and others, 2006). Taken together, in response to seizures more excitatory neurons are generated which are more rapidly integrated in the circuitry with some of them generating recurrent and excitatory feedback connections.

In our study we did however not find evidence of reduced epileptogenesis after suppressing seizure-induced neurogenesis by using radiation. In fact, radiated rats kindled more rapidly and displayed more excitable hippocampal networks. Therefore, the results of our study imply that if seizure-generated neurons contribute to epileptogenesis their role is probably only minimal and could even be protective. However, some important questions remain unanswered and require further research.

Firstly, by radiating rats one day before the initiation of kindling we showed that seizure-induced neurogenesis did not play a major role in kindling epileptogenesis. However as radiation spares postmitotic neurons it could still be that immature neurons, generated during ongoing neurogenesis in the weeks before kindling, contribute to kindling epileptogenesis. As it takes approximately four to seven weeks until newborn neurons become indistinguishable from mature granule neurons (Kempermann and others, 2004), a delay of two months between radiation and kindling would exclude that immature neurons are still present at the start of kindling.

Secondly, we found that a single radiation dose of 8Gy was sufficient to suppress neurogenesis during kindling acquisition. However, neurogenesis was not permanently blocked as three weeks after radiation new

neuroblasts were generated. This recovery is most probably due to stimulatory effects of seizures. Radiation with two fractionated doses of 10 Gy, spaced by 24 hours, was shown to be sufficient for permanent suppression of neurogenesis even after pathological stimulation (Wojtowicz, 2006). Therefore in future studies it may be more advantageous to choose for fractionated doses, especially in case of a longer delay between the radiation and the initiation of kindling.

Thirdly, aberrant neurogenesis seems to be more pronounced in post-SE models and could therefore play a more prominent role in epileptogenic processes which lead to spontaneous seizures. In a study, done by Parent and colleagues, rats were radiated one day before PILO induced SE to suppress neurogenesis and to determine the contribution of seizure-generated neurons to mossy fiber sprouting (Parent and others, 1999). Although the same authors had previously shown that part of the newborn neurons generate aberrantly sprouted mossy fibers (Parent and others, 1997), radiation did not result in a reduction of mossy fiber sprouting. The authors hypothesized that mature granule neurons were responsible for most of the mossy fiber sprouting. However, the authors could not exclude the possibility that immature neurons, generated in the weeks before radiation, were responsible for aberrant mossy fiber sprouting. Also the effect of radiation on the development of spontaneous seizures was not investigated. Therefore, a future experiment in which neurogenesis is blocked by fractionated radiation several weeks before the induction of SE in rats may be very interesting. Histological studies should be done to confirm the suppression of seizure induced aberrant neurogenesis and video-EEG monitoring should be performed to assess the effect on the development on spontaneous seizures.

Fourthly, low-dose radiation is a rather unspecific tool to suppress neurogenesis and can evoke inflammation, cause microvascular changes and induce alterations in metabolic and signalling pathways (Monje and Palmer, 2003; Silasi and others, 2004). So, enhanced excitability and kindling rate after radiation-induced suppression of neurogenesis could be the result of aspecific side effects of radiation rather than a proof that seizure-induced neurogenesis is a protection mechanism against hyperexcitability. In order to make more definite conclusions about the role of neurogenesis in epileptogenesis other models for suppression of neurogenesis are mandatory. One alternative model in which neurogenesis can be selectively suppressed is currently under development at the “Laboratory for Neurodevelopment and Regeneration” (University of Michigan, USA) under the supervision of Dr. Jack Parent. In this laboratory a transgenic mouse has been created in which thymidine kinase is expressed under the control of the nestin promoter, which is selectively activated in NSC. Thymidine kinase metabolizes gangcyclovir into toxic nucleotides, so injection of gangcyclovir in adult nestin-thymidine kinase transgenic mice leads to selective apoptosis of adult NSC and subsequent suppression of neurogenesis. In this model there may be fewer aspecific side effects compared to radiation. So, it may be interesting to test whether in this transgenic model enhanced excitability during kindling epileptogenesis after suppression of neurogenesis is also evident. If so, this would further support the idea that seizure-induced neurogenesis is a protection mechanism against hyperexcitability. Consequently, strategies could be developed to stimulate seizure-induced neurogenesis, e.g. by local infusion of growth factors which stimulate neurogenesis such as IGF-1.

In stroke models it was found that lost CA1 pyramidal neurons can be regenerated by precursors that migrate from the posterior periventricular region (PPV) to the ischemically damaged CA1 region. In case of hippocampal sclerosis after PILO induced SE, the same migration of precursor cells from PPV to damaged CA1 and CA3 regions has been described but the precursor cells exclusively generated glial cells in the damaged regions. Unravelling the mechanisms behind pyramidal cell replacement in case of ischemia may lead to strategies to stimulate endogenous replacement of pyramidal neurons in case of hippocampal sclerosis and TLE. For example it was found that replacement of CA1 pyramidal neurons after ischemia can be dramatically enhanced by intraventricular infusion of bFGF and EGF (Nakatomi and others, 2002). So, local infusion of growth factor could be one possible strategy to stimulate endogenous repair of hippocampal networks in TLE. As already stated above, next to pyramidal neurons also other neuronal phenotypes such as hilar interneurons will need to be replaced. One study has demonstrated the generation of new inhibitory interneurons in adult hippocampus, indicating that endogenous replacement of lost interneurons may be possible (Liu and others, 2003). But again it needs to be stressed that even if lost neurons can be replaced by stimulating endogenous sources, coordinated integration of these new neurons will be a crucial and highly complex step.

REFERENCES

- Akiyama Y, Honmou O, Kato T, Uede T, Hashi K, Kocsis JD. 2001. Transplantation of clonal neural precursor cells derived from adult human brain establishes functional peripheral myelin in the rat spinal cord. *Exp Neurol* 167(1):27-39.
- Andsberg G, Kokaia Z, Bjorklund A, Lindvall O, Martinez-Serrano A. 1998. Amelioration of ischaemia-induced neuronal death in the rat striatum by NGF-secreting neural stem cells. *European Journal of Neuroscience* 10(6):2026-36.
- Behrstock S, Ebert A, McHugh J, Vosberg S, Moore J, Schneider B, Capowski E, Hei D, Kordower J, Aebischer P, Svendsen CN. 2005. Human neural progenitors deliver glial cell line-derived neurotrophic factor to parkinsonian rodents and aged primates. *Gene Ther* 13(5):379-88.
- Boison D, Scheurer L, Zumsteg V, Rulicke T, Litynski P, Fowler B, Brandner S, Mohler H. 2002. Neonatal hepatic steatosis by disruption of the adenosine kinase gene. *Proc Natl Acad Sci U S A* 99(10):6985-90.
- Chu K, Kim M, Jung KH, Jeon D, Lee ST, Kim J, Jeong SW, Kim SU, Lee SK, Shin HS, Roh JK. 2004. Human neural stem cell transplantation reduces spontaneous recurrent seizures following pilocarpine-induced status epilepticus in adult rats. *Brain Res* 1023(2):213-21.
- Fedele DE, Koch P, Scheurer L, Simpson EM, Mohler H, Brustle O, Boison D. 2004. Engineering embryonic stem cell derived glia for adenosine delivery. *Neurosci Lett* 370(2-3):160-5.
- Greber S, Schwarzer C, Sperk G. 1994. Neuropeptide Y inhibits potassium-stimulated glutamate release through Y2 receptors in rat hippocampal slices in vitro. *Br J Pharmacol* 113(3):737-40.
- Guttinger M, Fedele D, Koch P, Padrun V, Pralong WF, Brustle O, Boison D. 2005a. Suppression of kindled seizures by paracrine adenosine release from stem cell-derived brain implants. *Epilepsia* 46(8):1162-9.
- Guttinger M, Padrun V, Pralong WF, Boison D. 2005b. Seizure suppression and lack of adenosine A1 receptor desensitization after focal long-term delivery of adenosine by encapsulated myoblasts. *Exp Neurol* 193(1):53-64.
- Haberman RP, Samulski RJ, McCown TJ. 2003. Attenuation of seizures and neuronal death by adeno-associated virus vector galanin expression and secretion. *Nat Med* 9(8):1076-80.
- Huber A, Padrun V, Deglon N, Aebischer P, Mohler H, Boison D. 2001. Grafts of adenosine-releasing cells suppress seizures in kindling epilepsy. *Proc Natl Acad Sci U S A* 98(13):7611-6.
- Kempermann G, Jessberger S, Steiner B, Kronenberg G. 2004. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci* 27(8):447-52.
- Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-Pernaute R, Bankiewicz K, McKay R. 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418(6893):50-6.
- Klein SM, Behrstock S, McHugh J, Hoffmann K, Wallace K, Suzuki M, Aebischer P, Svendsen CN. 2005. GDNF Delivery Using Human Neural Progenitor Cells in a Rat Model of ALS. *Human Gene Therapy* 16(4):509-21.
- Lin EJ, Richichi C, Young D, Baer K, Vezzani A, During MJ. 2003. Recombinant AAV-mediated expression of galanin in rat hippocampus suppresses seizure development. *European Journal of Neuroscience* 18(7):2087-92.
- Liu S, Wang J, Zhu D, Fu Y, Lukowiak K, Lu YM. 2003. Generation of functional inhibitory neurons in the adult rat hippocampus. *J Neurosci* 23(3):732-6.
- Liu Y, Himes BT, Solowska J, Moul J, Chow SY, Park KI, Tessler A, Murray M, Snyder EY, Fischer I. 1999. Intraspinal delivery of neurotrophin-3 using neural stem cells genetically modified by recombinant retrovirus. *Exp Neurol* 158(1):9-26.
- Lu P, Jones LL, Snyder EY, Tuszynski MH. 2003. Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. *Experimental Neurology* 181(2):115-29.
- McCown TJ. 2006. Adeno-associated Virus-Mediated Expression and Constitutive Secretion of Galanin Suppresses Limbic Seizure Activity in Vivo. *Mol Ther* 14(1):63-8.

- Monje ML, Palmer T. 2003. Radiation injury and neurogenesis. *Curr Opin Neurol* 16(2):129-34.
- Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M. 2002. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* 110(4):429-41.
- Overstreet-Wadiche LS, Bromberg DA, Bensen AL, Westbrook GL. 2006. Seizures accelerate functional integration of adult-generated granule cells. *J Neurosci* 26(15):4095-103.
- Parent JM, Lowenstein DH. 2002. Seizure-induced neurogenesis: are more new neurons good for an adult brain? *Prog Brain Res* 135:121-31.
- Parent JM, Tada E, Fike JR, Lowenstein DH. 1999. Inhibition of dentate granule cell neurogenesis with brain irradiation does not prevent seizure-induced mossy fiber synaptic reorganization in the rat. *J Neurosci* 19(11):4508-19.
- Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH. 1997. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci* 17(10):3727-38.
- Pluchino S, Zanotti L, Rossi B, Brambilla E, Ottoboni L, Salani G, Martinello M, Cattalini A, Bergami A, Furlan R, Comi G, Constantin G, Martino G. 2005. Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. *Nature* 436(7048):266-71.
- Qian J, Colmers WF, Saggau P. 1997. Inhibition of synaptic transmission by neuropeptide Y in rat hippocampal area CA1: modulation of presynaptic Ca²⁺ entry. *J Neurosci* 17(21):8169-77.
- Richichi C, Lin EJ, Stefanin D, Colella D, Ravizza T, Grignaschi G, Veglianesi P, Sperk G, During MJ, Vezzani A. 2004. Anticonvulsant and antiepileptogenic effects mediated by adeno-associated virus vector neuropeptide Y expression in the rat hippocampus. *J Neurosci* 24(12):3051-9.
- Ryu JK, Kim J, Cho SJ, Hatori K, Nagai A, Choi HB, Lee MC, McLarnon JG, Kim SU. 2004. Proactive transplantation of human neural stem cells prevents degeneration of striatal neurons in a rat model of Huntington disease. *Neurobiology of Disease* 16(1):68-77.
- Scharfman HE, Goodman JH, Sollas AL. 2000. Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. *J Neurosci* 20(16):6144-58.
- Shapiro LA, Korn MJ, Ribak CE. 2005. Newly generated dentate granule cells from epileptic rats exhibit elongated hilar basal dendrites that align along GFAP-immunolabeled processes. *Neuroscience* 136(3):823-31.
- Silasi G, az-Heijtz R, Besplug J, Rodriguez-Juarez R, Titov V, Kolb B, Kovalchuk O. 2004. Selective brain responses to acute and chronic low-dose X-ray irradiation in males and females. *Biochemical and Biophysical Research Communications* 325(4):1223-35.
- Svendsen CN, Caldwell MA. 2000. Neural stem cells in the developing central nervous system: implications for cell therapy through transplantation. *Prog Brain Res* 127:13-34.
- Terman GW, Drake CT, Simmons ML, Milner TA, Chavkin C. 2000. Opioid modulation of recurrent excitation in the hippocampal dentate gyrus. *J Neurosci* 20(12):4379-88.
- Vescovi AL, Parati EA, Gritti A, Poulin P, Ferrario M, Wanke E, Frolichsthal-Schoeller P, Cova L, Arcellana-Panlilio M, Colombo A, Galli R. 1999. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Exp Neurol* 156(1):71-83.
- Westerlund U, Svensson M, Moe MC, Varghese M, Gustavsson B, Wallstedt L, Berg-Johnsen J, Langmoen IA. 2005. Endoscopically harvested stem cells: a putative method in future autotransplantation. *Neurosurgery* 57(4):779-84.
- Wojtowicz JM. 2006. Irradiation as an experimental tool in studies of adult neurogenesis. *Hippocampus* 16(3):261-6.

Yan J, Welsh AM, Bora SH, Snyder EY, Koliatsos VE. 2004. Differentiation and tropic/trophic effects of exogenous neural precursors in the adult spinal cord. *J Comp Neurol* 480(1):101-14.

Zini S, Roisin MP, Langel U, Bartfai T, Ben-Ari Y. 1993. Galanin reduces release of endogenous excitatory amino acids in the rat hippocampus. *Eur J Pharmacol* 245(1):1-7.

SUMMARY

The treatment of choice for epilepsy is the chronic administration of antiepileptic drugs (AED). However, a high fraction of patients with temporal lobe epilepsy (TLE) continues to have uncontrolled seizures and these patients are called refractory. Hippocampal sclerosis is believed to be the most important pathological substrate for TLE. Resection of the sclerotic tissue is the most successful alternative treatment option for refractory TLE. However, a fraction of the refractory TLE patients cannot undergo resective surgery because the seizure focus cannot be identified or because of a possible risk for functional loss (language and memory). Moreover, a proportion of the TLE patients continues or restarts to have seizures after surgery. For those patients it is important to develop alternative treatments. One possible alternative treatment is cell therapy. In this study we evaluated the feasibility of two different cell therapy strategies: cell transplantation and modulation of endogenous neurogenesis.

Cell transplantation has already been performed in different models for TLE and this for two different purposes: restoration of epileptic neuronal networks and/or local delivery of seizure-suppressing substances. In both transplantation approaches fetal cells have been most frequently used as cell source. Ethical and practical issues coupled to the use of fetal cells have urged the search for alternative donor cell types. Based on their expansion capacity and their ability to generate different neural phenotypes, neuropotent stem cells may be ideal candidates to replace fetal brain cells as cell source for transplantation in neurodegenerative diseases. Most interesting are adult-derived neuropotent stem cells which may lead to autologous transplantation strategies, free of ethical and immunorejection problems.

In the Laboratory for Clinical and Experimental Neurophysiology (LCEN) at Ghent University Hospital two different adult-derived stem cell types were isolated and expanded in culture. Neural stem cells (NSC) were derived from the subventricular zone (SVZ) of the brain and multipotent adult progenitor cell (MAPC)-like cells from the bone marrow of adult rats. Both cell types were compared as to their efficiency to generate mature neural phenotypes after exposure to three neural differentiation protocols. Cell morphology was evaluated and the expression of neuronal markers (β 3-tubulin, NF-200, tau), an astrocyte marker (GFAP) and oligodendrocyte markers (RIP, MBP) was screened using real-time PCR and immunocytochemistry before and after differentiation. In contrast to the NSC, the MAPC-like cells did not reproducibly adopt neural morphology nor expressed all differentiation markers. This led us to conclude that MAPC-like cells were inferior to NSC in their neuropotency. In fact, in line with other recent evidence, our results suggest that the adoption of neural morphology and the expression of neural markers by non-neural cells can be a non-specific phenomenon rather than proof of neuropotency. Based on the results of these *in vitro* experiments, we chose to use adult-derived NSC for future neural transplantation experiments.

Cell grafting in animal models for TLE has been successful in reversing some pathological alterations and suppressing excitability. However, before extrapolations to the human situation can be made, the long-term effect of transplantation on spontaneous seizures needs to be investigated more thoroughly in chronic TLE

models. The intrahippocampal kainic acid (KA) rat model is a chronic post-status epilepticus (SE) model for TLE. This model displays typical hippocampal sclerosis and spontaneous epileptic seizures, mostly originating from the hippocampus ipsilateral to the KA lesion. Due to the focal nature of the initial damage and seizure origin, the intrahippocampal KA model is very interesting for evaluating local cell transplantation. So far, characterization of spontaneous seizures in this model had not been performed. In this study, we monitored the intrahippocampal KA model for up to 42 weeks with video-EEG recording to determine characteristics of spontaneous seizure such as seizure duration, severity and frequency. Overall, we found that the characteristics of spontaneous seizures were comparable to other post-SE models. Intrahippocampal KA injection was very efficient as all treated rats developed SE and subsequent spontaneous seizures. In 87 % of the rats the first spontaneous seizure was detected during the first eight weeks after KA injection. Seizures lasted on average about 1 minute and 34 % of all seizures were partial without a clear behavioural component. Seizures occurred in a circadian pattern with peak occurrence during the daytime. Seizure frequency was rather low and highly variable among rats, but increased with time. This model could be useful to screen the effect of cell transplantation on spontaneous seizures given that extensive monitoring on a high number of rats could be performed.

We derived NSC from the subventricular zone (SVZ) of adult green fluorescent protein (GFP)-reporter mice and transplanted them into the sclerotic hippocampus of the intrahippocampal KA model, three days and three weeks after KA injection. Survival, dispersion and differentiation of the transplanted NSC were evaluated three and six weeks after transplantation. To determine the effect of the KA lesion on the outcome parameters, cells were also transplanted in normal hippocampus of non-epileptic rats. Three weeks after transplantation, a small fraction (about 1%) of the transplanted cells was found in the host brain. This percentage of detected cells remained relatively stable for at least six weeks after transplantation. The presence of the lesion had no major influence on the survival of the transplanted cells but we found that survival rate was significantly higher if cells were transplanted three days compared to three weeks after the KA lesion. In the sclerotic hippocampus cells remained in rather dense clusters in contrast to the intact hippocampus, in which cells aligned along hippocampal fracture planes. The transplanted NSC differentiated mainly towards astrocytes both in the intact and the sclerotic hippocampus. This astrocytic differentiation was clearly stimulated upon transplantation in the sclerotic hippocampus, indicating that NSC contribute to the gliotic response to the KA lesion. Although a small fraction of the transplanted NSC differentiated towards neuronal cells, there was no indication for neuronal replacement or repair of damaged circuitry. Based on their stable survival, NSC could be used for local delivery of seizure suppressing substances in sclerotic hippocampus.

A second potential cell therapy approach for TLE entails the modulation of endogenous neurogenesis. In the dentate gyrus of the hippocampus, excitatory granule neurons are continuously generated throughout life. In different models for TLE, granule cell neurogenesis is highly affected during epileptogenesis. The number of newborn granule neurons is enhanced 3- to 10-fold in response to seizures. A proportion of the immature neurons displays inappropriate synaptic connectivity and integrates at ectopic locations. Some of these ectopic

granule neurons seem to be incorporated in epileptic networks and display epileptic bursting behaviour. Based on these observations several authors have suggested that neurogenesis contributes to epileptogenesis. To further explore the role of aberrant neurogenesis in response to seizures we used low-dose, whole brain radiation to suppress the generation of new neurons during epileptogenesis in the kindling model for TLE. Although new neuron formation in the hippocampus was dramatically suppressed during kindling of the radiated rats, this did not prevent the rats from becoming fully epileptic. In fact, the threshold to evoke a seizure was decreased and the kindling rate increased in radiated rats. Further research is mandatory to determine whether this increased excitability is the result of suppressing seizure-induced neurogenesis or a non-specific side effect of the radiation. This study indicated that seizure-induced neurogenesis plays no major role in the epileptogenesis. So, blocking the generation of seizure-induced neurons has little potential to become a cell therapy option for TLE.

SAMENVATTING

Chronische toediening van anti-epileptica is de standaardbehandeling voor epilepsie. Maar in het geval van temporale kwab epilepsie (Temporal Lobe Epilepsy, TLE) blijft een groot percentage van de patiënten aanvallen vertonen. Deze patiënten hebben refractaire temporale kwab epilepsie. Hippocampale sclerose is het belangrijkste pathologische substraat voor TLE. De chirurgische verwijdering van het sclerotisch weefsel is de meest succesvolle alternatieve behandeling voor refractaire TLE. Er is echter een deel van de patiënten bij wie chirurgische wegname niet mogelijk is omdat de aanvalsfocus niet kan geïdentificeerd worden of omdat er een risico bestaat voor functie-uitval. Bovendien blijft een deel van de geopereerde TLE patiënten aanvallen vertonen of beginnen de aanvallen opnieuw na een aanvalsvrije periode. Voor al deze patiënten is het belangrijk dat gezocht wordt naar alternatieve behandelingen. Eén van de mogelijke alternatieve behandelingen is celtherapie. In deze studie evalueerden wij de toepasbaarheid van twee verschillende celtherapie behandelingen: celtransplantatie en modulatie van endogene neurogenese.

Celtransplantatie is reeds eerder uitgevoerd bij verschillende diermodellen voor TLE en dit voor twee doeleinden: herstel van epileptische neuronale netwerken en/of lokale secretie van aanvalsonderdrukkende stoffen. De meest frequent gebruikte celbron voor beide benaderingen zijn foetale cellen. Ethische en praktische problemen, gekoppeld aan het gebruik van foetale cellen, hebben echter aanleiding gegeven tot een zoektocht naar alternatieve donor celtypes. Op basis van hun expansievermogen en hun capaciteit om verschillende neuronale fenotypes te genereren, zouden neuropotente stamcellen ideale alternatieve celtypes kunnen zijn. Meest interessant zijn de neuropotente stamcellen afkomstig van volwassen weefsels, omdat deze kunnen leiden tot autologe transplantatie strategieën, vrij van ethische kwesties of afstotingsproblemen.

In het Laboratorium voor Klinische en Experimentele Neurofysiologie (LKEN) van het Universitair Ziekenhuis van Gent werden twee stamceltypes, afkomstig van volwassen weefsels van de rat, geïsoleerd en geëxpandeerd in cultuur. Neurale stamcellen (NSC) werden geïsoleerd uit de subventriculaire zone (SVZ) van de hersenen en multipotente adulte progenitor cel (MAPC)-achtige cellen uit het beenmerg van volwassen ratten. Beide celtypes werden onderworpen aan drie neuronale differentiatie protocollen en vergeleken in hun efficiëntie om volwassen neurale fenotypes te produceren. Celmorfologie werd vergeleken en de expressie van neuronale merkers (β 3-tubulin, NF-200, tau), een astrocytaire merker (GFAP) en oligodendrocytaire merkers (RIP, MBP) werd geëvalueerd met behulp van real-time PCR en immunocytochemie voor en na differentiatie. In tegenstelling tot de NSC waren de MAPC-achtige cellen niet in staat om op een reproduceerbare manier neuronale en gliale morfologieën aan te nemen of om alle differentiatie markers tot expressie te brengen. Op basis van deze resultaten concludeerden we dat de neuropotentie van MAPC-achtige cellen lager was dan deze van NSC. Bovendien geven onze resultaten aan dat, in analogie met andere recente studies, de inductie van een neuronale morfologie en de expressie van neurale merkers een specifiek fenomeen kan zijn in plaats van een bewijs voor neuropotentie. Aan de hand van de resultaten van deze *in vitro* experimenten verkozen we adulte NSC te gebruiken in de neurotransplantatie experimenten.

Studies hebben aangetoond dat celtransplantatie in verschillende diermodellen voor TLE kan leiden tot een gedeeltelijke omkering van pathologische veranderingen en een verlaging van de exciteerbaarheid. Maar vooraleer extrapolaties kunnen gemaakt worden naar TLE patiënten zijn meer uitgebreide en lange-termijn studies in chronische modellen voor TLE noodzakelijk. Het intrahippocampaal kainaat (KA) model is een chronisch, post-status epilepticus (SE) model voor TLE. Dit rat model vertoont typische hippocampale sclerose en spontane epileptische aanvallen die meestal beginnen in de hippocampus aan dezelfde kant van de KA laesie. De focale aard van de initiële schade en het aanvalsbegint maakt dit model zeer geschikt voor de evaluatie van lokale celtransplantatie. Tot nog toe werden de spontane aanvallen in dit model nog niet gekarakteriseerd. Wij volgden het intrahippocampaal KA model tot 42 weken na KA inspuiting met behulp van video-EEG monitoring om kenmerken zoals de duur, de ernst en de frequentie van de spontane aanvallen te bepalen. Over het algemeen vonden we dat de aanvalskarakteristieken vergelijkbaar waren met andere post-SE modellen. Intrahippocampale KA injectie was zeer efficiënt aangezien alle behandelde ratten een SE en daaropvolgende spontane aanvallen ontwikkelden. In 87% van de ratten werd een eerste aanval gedetecteerd in de eerste acht weken na SE. De epileptische aanvallen duurden gemiddeld ongeveer één minuut en 34% van de aanvallen waren partieel zonder een duidelijke gedragscomponent. De aanvallen volgden een circadiaan patroon met meer aanvallen overdag. De aanvalsfrequentie was eerder laag en sterk variabel tussen de ratten onderling maar nam toe met de tijd. Dit model zou gebruikt kunnen worden om het effect van celtransplantatie op spontane aanvallen uit te testen op voorwaarde dat een groot aantal ratten op een uitgebreide manier gevolgd wordt met behulp van video en EEG.

Neurale stamcellen werden geïsoleerd van de SVZ van volwassen “green fluorescent protein (GFP)”-reporter muizen en getransplanteerd in de sclerotische hippocampus van het intrahippocampaal KA model, drie dagen en drie weken na KA injectie. Drie en zes weken na transplantatie werden de overleving, de dispersie en de differentiatie van de getransplanteerde NSC geëvalueerd. Om het effect van de laesie op deze uitkomstparameters te bepalen werden de cellen ook getransplanteerd in de intacte hippocampus van niet-epileptische ratten. Drie weken na transplantatie werd een klein deel van de getransplanteerde cellen (ongeveer 1%) teruggevonden in de hersenen van de gastheer. Dit percentage gedetecteerde cellen bleef stabiel voor minstens zes weken na transplantatie. De aanwezigheid van de laesie had geen duidelijke invloed op de overleving van de getransplanteerde cellen, maar we vonden wel dat de overleving significant hoger was indien cellen getransplanteerd werden drie dagen in plaats van drie weken na de KA laesie. In de sclerotische hippocampus bleven de cellen in dichte clusters terwijl in de intacte hippocampus de cellen meer verspreid de scheidingsvlakken van de hippocampus aflijnden. Zowel in de intacte als in de sclerotische hippocampus differentieerden de getransplanteerde NSC vooral naar astrocyten. De astrocytaire differentiatie was duidelijk gestimuleerd na transplantatie in de sclerotische hippocampus, wat erop wijst dat NSC bijdragen tot de gliotische respons. Hoewel een klein deel van de getransplanteerde NSC differentieerde naar neuronale cellen was er geen indicatie dat afgestorven neuronen vervangen werden of dat beschadigde netwerken hersteld werden.

Een tweede mogelijke celtherapie strategie voor TLE bestaat erin de endogene neurogenese te moduleren. In de dentate gyrus van de hippocampus worden doorheen het leven voortdurend nieuwe korrelcellen gevormd. In verschillende diermodellen voor TLE is korrelcel neurogenese sterk beïnvloed tijdens de epileptogenese. Het aantal nieuwgeboren korrelcellen is drie tot tien keer hoger na epileptische aanvallen. Een deel van deze jonge neuronen vormt abnormale synaptische connecties en integreert op abnormale (ectopische) locaties. Enkele van deze ectopische korrelcellen lijken geïncorporeerd te zijn in epileptische netwerken en vertonen epileptische ontladingsactiviteit. Op basis van deze bevindingen werd door verschillende auteurs verondersteld dat neurogenese bijdraagt tot epileptogenese. Om verder de rol van abnormale neurogenese in respons op epileptische aanvallen te onderzoeken, stelden we de hersenen van ratten bloot aan een lage dosis bestraling om zo de vorming van nieuwe neuronen te verhinderen tijdens epileptogenese in het kindling model voor TLE. Hoewel de productie van nieuwe neuronen in de hippocampus zeer sterk onderdrukt was tijdens het kindling proces, verhinderde dit niet dat de bestraalde ratten volledig gekindeld raakten. Meer nog, de drempel om een aanval uit te lokken was lager en de kindling snelheid hoger na bestraling. Verder onderzoek is echter nodig om uit te zoeken of deze verhoogde exciteerbaarheid het gevolg is van de onderdrukking van aanvalsgeïnduceerde neurogenese of een specifiek neveneffect van de bestraling. Deze studie geeft echter wel aan dat aanvalsgeïnduceerde neurogenese geen cruciale rol speelt in epileptogenese. Bijgevolg zal de onderdrukking van aanvalsgeïnduceerde neurogenese hoogstwaarschijnlijk geen celtherapie worden voor TLE.

L'épilepsie est traitée de façon classique avec des médicaments antiépileptiques. Néanmoins, chez une grande partie des patients souffrant d'une sclérose du lobe temporal, se manifeste une fréquence élevée de crises épileptiques. La sclérose de l'hippocampe est considérée comme la base pathologique de l'épilepsie temporale. C'est pourquoi la résection chirurgicale de ce tissu sclérotique reste, jusqu'à présent, le traitement alternatif avec le plus grand pourcentage de réussite. Cependant, une partie des patients ne peut pas être opérée, pour différentes raisons: il se peut que la localisation du foyer épileptique soit insuffisante, ou que l'intervention implique un risque de perte de fonctions cognitives importantes. En outre, une partie des patients récidive après une intervention chirurgicale. Pour tous ces patients, le développement de nouveaux traitements thérapeutiques valables peut avoir une signification clinique importante. Une possibilité du sujet de recherche intensive est la thérapie cellulaire. Le sujet de cette dissertation comprend l'évaluation de deux types de thérapies cellulaires: la transplantation cellulaire et la modulation de la neurogénèse endogène.

Jusqu'à présent, des cellules ont été transplantées dans différents modèles animaux d'épilepsie temporale avec des buts différents. La restauration des circuits épileptiques neuronaux et la délivrance de substances antiépileptiques de façon locale étaient les points d'intérêts les plus importants. Dans ces deux derniers cas, les cellules fœtales sont les plus fréquemment utilisées comme source de cellules. Dû aux contraintes éthiques et pratiques, il s'avérerait nécessaire de trouver d'autres ressources de cellules, n'ayant pas les mêmes contraintes que les cellules fœtales. Dans ce contexte, les cellules souches capables de générer des cellules neuronales peuvent être des candidats excellents pour des transplantations neurales dans les maladies neurodégénératives, car elles ont de très bonnes capacités d'expansion et peuvent se développer en phénotypes neuronaux différents. Les cellules souches dérivées des tissus adultes forment une source très intéressante, car elles peuvent amener au développement des stratégies de transplantation neuronales autologues. De cette façon, des problèmes éthiques et des réactions immunitaires sont évités.

Au Laboratoire de Neurophysiologie Clinique et Expérimentale (LNCE) de l'hôpital universitaire de Gand, deux types de cellules souches, provenant du tissu adulte du rat, ont été isolées et mises en culture. D'une part, des cellules souches neuronales (NSC) ont été dérivées de la zone subventriculaire (SVZ) du cerveau, d'autre part des cellules qui ressemblent aux cellules progénitrices adultes multipotentes (MAPC-like) ont été isolées de la moelle de rats adultes. Les deux types de cellules ont été exposés à trois protocoles de différenciation neuronale différents. Ensuite, leur capacité de générer des cellules avec un phénotype neuronal a été évaluée. On a comparé la morphologie des cellules et l'expression des marqueurs neuronaux (beta3-tubuline, NF-200 et tau), du marqueur astrocytaire (GFAP) et des marqueurs oligodendrocytaires (RIP, MBP) a été identifiée à l'aide de RT-PCR et d'immunocytochimie avant et après l'exposition au protocole spécifique. Quant aux NSC, les trois protocoles de différenciation ont généré des cellules avec les caractéristiques morphologiques de neurones ou de cellules gliales. En prime, l'expression de tous les gènes et antigènes neuronaux était élevée en conséquence de la différenciation. Les cellules MAPC-like développaient une morphologie semblable aux neurones dans seulement un des trois protocoles de différenciation. En plus, ces cellules exprimaient

seulement deux marqueurs neuronaux (beta3-tubuline et NF-200) et ne montraient pas d'expression augmentée après la différenciation. En outre, l'expression du marqueur neuronal (tau) et des marqueurs gliaux (GFAP, RIP et MPB) n'était pas détectée. L'incapacité de fournir des cellules neurales de façon reproductible nous a amené à conclure que les cellules MAPC-like sont inférieures aux NSC dans leur capacité de produire des cellules neurales adultes. En outre, ces résultats sont cohérents avec d'autres résultats scientifiques dans ce domaine, montrant que l'adoption d'une morphologie semblable aux neurones et l'expression de marqueurs neuronaux pourraient plutôt être un phénomène aspécifique qu'un témoignage du développement neuronal. Basé sur ces résultats in vitro, nous avons choisi de continuer nos expériences de transplantation avec des NSC adultes d'origine cérébrale.

Précédemment, des expériences scientifiques de transplantation cellulaire ont paru un succès, car il s'est avéré que dans quelques modèles d'épilepsie temporale certains changements pathologiques ont été partiellement rétablis. En plus, dans d'autres modèles, tels que des modèles de crises épileptiques évoquées, l'excitabilité neurale était réduite. Cependant, avant que des extrapolations à la situation humaine puissent être faites, l'effet à long terme de la transplantation sur des crises spontanées doit être étudié plus à fond dans les modèles chroniques de TLE.

Le modèle de kainate (KA) intrahippocampal est un modèle post état-de-mal (SE) de l'épilepsie du lobe temporal. Ce modèle montre la sclérose hippocampale typique et les crises épileptiques spontanées, provenant la plupart du temps de l'hippocampe ipsilatéral à la lésion de KA. En raison de la nature focale du dommage initial et de l'origine des crises épileptiques, le modèle de KA intrahippocampal est très intéressant pour évaluer la transplantation locale des cellules. Jusqu'ici, la caractérisation des crises spontanées dans ce modèle n'avait pas encore été réalisée. Dans cette étude, nous avons surveillé le modèle de KA intrahippocampal pendant 42 semaines avec l'enregistrement de vidéo et d'EEG pour déterminer des paramètres habituels de crises spontanées tels que la durée, la sévérité et la fréquence des crises. Nous avons constaté que les caractéristiques des crises spontanées étaient comparables à d'autres modèles post-SE. L'injection de KA intrahippocampal était très efficace alors que tous les rats traités ont développé SE et des crises spontanées ultérieures. Chez 87 % des rats, la première crise spontanée a été détectée pendant les huit premières semaines après l'injection de KA. La crise dure en moyenne environ 1 minute et 34 % de toutes les crises épileptiques étaient partielles sans généralisation secondaire. Les crises se sont produites dans un modèle circadien avec l'occurrence maximale pendant la journée. Généralement, la fréquence des crises épileptiques était plutôt basse et fortement variable parmi les rats. Seulement une petite fraction des rats (3 sur 15, 20 %) a montrée plus de trois crises en moyenne par jour. Avec le temps, une augmentation générale de la fréquence des crises a été observée. Ce modèle pourrait être employé pour examiner l'effet de la transplantation de cellules sur la suppression des crises spontanées, bien que la surveillance étendue et un nombre élevé de rats soient exigés.

Des NSC, dérivées du SVZ de souris protéine fluorescente verte (GFP) transgéniques adultes, ont été transplantées dans l'hippocampe sclérotique du modèle de KA intrahippocampal, à trois jours et à trois

semaines après l'injection de KA. La survie, la dispersion et la différenciation du NSC ont été évaluées trois et six semaines après leur transplantation. Pour déterminer l'effet de la lésion de KA sur les paramètres observés, des NSC ont également été transplantées dans l'hippocampe normal des rats non-épileptiques. Trois semaines après la transplantation, une petite fraction (environ 1 %) des cellules transplantées a été détectée dans le cerveau. Cette fraction n'a pas changé de manière significative pendant au moins six semaines. La présence de la lésion n'a eu aucune influence principale sur la survie des cellules transplantées. Néanmoins, nous avons constaté qu'un retard de trois jours entre la lésion et la transplantation a favorisé de manière significative la survie en comparaison d'un retard de trois semaines. Dans l'hippocampe sclérotique, les NSC sont demeurées dans des faisceaux plutôt denses contrairement à l'hippocampe intact, où les cellules sont alignées le long des plans de rupture hippocampals. Les NSC s'étaient différenciées principalement vers des astrocytes et cette différenciation astrocytique a été clairement stimulée par la transplantation dans l'hippocampe sclérotique, indiquant que les NSC contribuent à la réponse gliale. Bien qu'une petite fraction des cellules transplantées ait exprimé les marqueurs neuronaux, il n'y avait aucune indication pour le remplacement ou la réparation neuronale des circuits endommagés. Basé sur leur survie stable, les NSC pourraient être employées pour la délivrance locale des substances supprimant les crises épileptiques dans l'hippocampe sclérotique.

Une deuxième approche potentielle de thérapie cellulaire pour TLE consiste à moduler la neurogénèse endogène. Dans le gyrus denté de l'hippocampe, les cellules granulaires sont produites sans interruption durant toute la vie. Dans différents modèles pour TLE, la neurogénèse de cellules granulaires est fortement affectée pendant l'épileptogénèse. Le nombre de neurones granulaires nouveau-nés est augmenté de 3 à 10 fois. Une proportion des neurones immatures montre la connectivité synaptique inadéquate et ils s'incorporent aux endroits ectopiques. Certains de ces cellules granulaires ectopiques semblent être incorporées dans les réseaux épileptiques. Basé sur ces observations, plusieurs auteurs ont supposé que la neurogénèse contribue à l'épileptogénèse. Pour explorer de plus le rôle de la neurogénèse anormale en réponse aux crises, nous avons utilisé une dose d'irradiation basse pour supprimer la génération de nouveaux neurones granulaires pendant l'épileptogénèse dans le modèle de kindling pour TLE. Bien que la formation de nouveaux neurones dans l'hippocampe ait été nettement supprimée pendant le kindling de rats irradiés, ce fait n'a pas empêché les rats de devenir entièrement épileptiques. En fait, le seuil pour évoquer une crise a été diminué et le taux de kindling a été augmenté chez les rats irradiés. Il reste à déterminer si cette excitabilité accrue est le résultat de la suppression de la neurogénèse ou un effet secondaire et non spécifique de l'irradiation. Cette étude a indiqué que la neurogénèse induite par les crises ne joue aucun rôle principal dans l'épileptogénèse. Ainsi, le blocage de la génération des neurones induits par les crises a peu de chance de devenir une option de thérapie cellulaire pour TLE.

DANKWOORD-ACKNOWLEDGEMENTS

Naar het schrijven van dit hoofdstuk heb ik het meest uitgekeken. Ten eerste, omdat dit betekent dat mijn thesis bijna af is. En ten tweede, omdat ik eindelijk de kans krijg om iedereen, die heeft bijgedragen aan het realiseren van dit proefschrift, eens te bedanken.

Eerst en vooral wil ik mijn promotor, **Prof. dr. Paul Boon**, ongelofelijk danken. Paul, u was voor mij alles wat het woord promotor inhoudt. Ik kon bij u altijd terecht voor mijn wetenschappelijke maar ook met persoonlijke problemen. Uw aanhoudend enthousiasme stuwde me vooruit en op een gegeven ogenblik was u het die me overtuigde niet op te geven. Bedankt om mij te laten proeven van de “geneugten” van een wetenschappelijk congres en mij te introduceren bij enkele van uw vooraanstaande collega’s.

Ik ben ook **Prof. dr. Jean Plum**, mijn co-promotor, en **Prof. dr. Bart Van de Kerckhove** zeer dankbaar. Dankzij hen kon ik beschikken over de nodige logistiek voor de isolatie en de kweek van de stamcellen. Tevens stonden zij mij altijd bij met raad en daad bij tal van technische moeilijkheden en betrokken ze me bij de wetenschappelijke activiteiten van hun labo.

Mijn thesis zou ook zeker niet geworden zijn wat hij nu is zonder de hulp **Prof. dr. Wytse Wadman**, ongetwijfeld de slimste mens die ik tot nu toe ontmoet heb. Wytse, ik kijk echt enorm naar u op en ik wil u ontzettend danken voor alle steun bij het opzetten van de experimenten, de data-analyse en het schrijven van de manuscripten. De verschillende bezoeken aan uw labo in het Swammerdam Instituut waren voor mij bijzonder leerrijk en stimulerend. Ik wil ook uw medewerkers **Hans Van Hooft, Pascal Chameau, Jan Gorter en Taco Werkman** bedanken voor de leuke babbels en hun hulp bij mijn “Amsterdamse experimenten”. Ik keek ook altijd uit naar de meerdaagse excursies want dat betekende dat ik mocht logeren ten huize Wadman waar **Hannah** altijd de lekkerste gerechten had klaargemaakt. Hannah bedankt voor de goede zorgen, de gastvrijheid en de vele gezellige momenten.

Verder wil ik ook mijn collega’s van het Laboratorium voor Klinische en Experimentele Neurofysiologie (LKEN) bedanken voor alle hulp en de leuke sfeer. **Annelies**, ik vond het ontzettend fijn om met jou samen te werken en ik kijk al uit naar de experimenten die we in de komende jaren zullen uitvoeren. **Kristl**, bedankt voor de vele verbeteringen en herlezingen van mijn artikels alsook de suggesties voor nieuwe experimenten en mooiere uitnodigingen. **Tim en Tine**, bedankt om samen met mij aan de video-EEG opstellingen te werken en deze verder te optimaliseren. **Deborah**, ik vond het zeer fijn om u te begeleiden tijdens uw thesis en ik vind dat je schitterend werk geleverd hebt. **Riëm** bedankt om mij te helpen met de résumé. Ook bedankt aan alle andere collega (ex-)doctoraatstudenten van het LKEN (**Veerle, Liesbeth, Riem, Evelien, Dimitri, Petra, Hans, Peter, Ronald, Sara, Pieter, Benjamin en Stefanie**) voor al hun hulp en de vele leuke momenten. **Isabel**, bedankt om voor mij faxmachine te spelen. **Jeroen**, dank zij jou raken veel van onze computerproblemen eindelijk opgelost. **Corine**, bedankt om alle administratieve zaken in goede banen te leiden. Het is voor ons echt een verademing sinds jij er bent.

In het labo voor Immunologie leerde ik tal van doctoraatstudenten en ander wetenschappelijk personeel kennen met wie het zeer aangenaam werken was. Onder hen wil ik vooral **Jef Pinxteren** en **David Craeye** bedanken voor de plezierige momenten binnen maar ook buiten het labo. Dankzij hen was het cellen splitsen tijdens de late uren en in het weekend veel aangenamer. Jef en David, na jullie vertrek was de sfeer in het labo nooit meer dezelfde. Gelukkig was **Frank Timmermans** er waarmee ik urenlang kon babbelen over wetenschap en alle mogelijke valkuilen, *t'è woa wè*. Ook een woordje van dank aan **Prof. Dr. Georges Leclercq** die er voor zorgde dat ik kon deelnemen aan de wetenschappelijke discussies op vrijdagmiddag en met wie ik stimulerende babbels had over een mogelijke toekomst als wetenschapper. Ook bedankt aan **Magda De Smedt** die mij inwijdde in alles wat te maken had met antilichamen en kleuringen. Ik wil ook al de andere mensen van het labo voor immunologie bedanken (**Katia, Inge, Kaatje, Femke, Tina, Veronique, Greet, Evelien, Tom,...**) omdat ik hen altijd kon storen en ze altijd bereid waren mij te helpen.

Er zijn ook nog een aantal andere mensen binnen het UZ Gent die ik zou willen bedanken voor hun bijdrage aan dit proefschrift. **Anouk**, dank voor al uw hulp en advies bij de immunohistochemie en het aanleren van confocale microscopie. Deze technieken zijn cruciaal gebleken voor de meeste van mijn experimenten. Ook een speciaal woordje van dank voor **Prof. Romain Lefebvre** die het oorspronkelijk mee mogelijk maakte dat het LKEN kon beschikken over de noodzakelijke lokalen en licenties. **Prof. Luc Leybaert** voor het ter beschikking stellen van apparatuur en personeel. **Cyriel en Dirk** voor de hulp bij het vervaardigen van de meet- en stimulatie-apparatuur en **Roland** voor alle technische hulp op ander gebied. **Prof. Rik Achten** en **Pieter Vandemaele** voor het nemen van de NMR scans van de rattenhersenen en **dr. Tom Boterberg** voor het bestralen van de ratten. Verder wil ik ook **dr. Ingrid Van Overbeke** en de andere mensen van het animalarium (**Lut, Martin, Chantal, Marrit**) bedanken voor de goede verzorging van de ratjes, ondanks het feit dat epileptische ratjes niet bepaald de liefste zijn.

De contacten in het UZ waren niet altijd van wetenschappelijke maar ook soms van sportieve aard. Tijdens mijn thesis kwam ik, via Jef Pinxteren, in contact met een groepje doctorandi en wetenschappelijk personeel die wekelijks een voetbalmatchke speelden op de terreinen van het UZ. Na een aantal vergaderingen in café “De Pinte” groeide dit groepje al snel uit tot een heuse voetbalploeg, genaamd PIMEK. Met PIMEK namen we deel aan nationale en internationale toernooien en organiseerden we ook lokale festiviteiten zoals een fuif en een kaas-en wijnavond. Bedankt **David, Dave, Detlev, Piet, Bruce, Joris, Feetje, Jan, Andy, Tony, Bart, Jef** voor de vele leuke en ontspannende momenten.

I would also like to thank our Swedish collaborators from the University of Göteborg. **Prof. Dr. Elinor Ben-Menachem**, for sharing her inspiring thoughts on neurogenesis and epilepsy and for bringing us in contact with **Prof. dr. Peter Eriksson**. I will always remember Peter for his enthusiasm, vision and energy and I am proud to have personally known him. Next, I also want to thank **Ann-Marie Allborn** and **Åsa Persson**, working at Peter's lab, as they helped a lot analyzing the neurogenesis data.

Also many thanks to **Prof. Dr. Catherine Verfaillie** and her coworkers (**Aernoud, Juehua, Beatriz, Terry, Carlos, Fernando, ...**) for the very interesting training on the culture and differentiation of MAPC. Besides working hard, I really enjoyed getting to know you all.

Verder zou ik ook een aantal mensen willen bedanken die misschien niet rechtstreeks hebben bijgedragen tot het realiseren van dit eindwerk maar waarvan ik zeker weet dat ze mijn interesse voor onderzoek hebben gestimuleerd. Bedankt **Prof. dr. Frans Jacobs, Prof. dr. Jacques Caemaert en Nico Peiren** om mij te begeleiden tijdens mijn eerste stapjes in het onderzoek. Ook een speciaal woordje van dank aan **Dirk Dobbels** die mij tijdens mijn licentiaatthesis en in het begin van mijn doctoraat geholpen heeft met het bouwen van een stereotactisch kader en speciaal aangepaste neurofysiologie kooien.

Ik dank de **leden van de examencommissie, leescommissie en begeleidingscommissie** van dit doctoraatswerk.

Ik dank daarenboven **Prof. dr. Jean-Louis Pannier**, decaan van de Faculteit Geneeskunde en Gezondheidswetenschappen en de heer **Christian Maes**, administratief secretaris.

Ik wil ook mijn vrienden bedanken die altijd bereid waren om mij te helpen ontspannen. **Griet, Steven, Liesbet, Delphine, Thierry, Heidi, Fabrice en Hannelore**, we zien mekaar wat minder nu ik zo ver woon maar ik geniet nu nog meer van de momenten dat we samen zijn. **Mieke, Jerry, Anneleen, Stoffe, Sofie, Reinout, Carl en Jozefien (alias “de Biologen”)**, ik heb echt genoten van onze “donderdagdagen” en hopelijk kunnen we in de toekomst regelmatig blijven afspreken want jullie zijn stuk voor stuk prachtige mensen. **Karoline, Nicolas, Koen, Sofie, Steven, Katrien, Jimmy, Tine, Kristof, Kristien en Jan**, ik heb jullie in de voorbije drie jaar leren kennen als lieve en toffe vrienden. **Kristien**, ik wil jou in het bijzonder bedanken voor het controleren en verbeteren van mijn résumé.

Tenslotte wil ik ook mijn ouders, schoonouders, familie en schoonfamilie bedanken. **Ma en Pa, Hugo en Mariake** bedankt voor jullie onvoorwaardelijke zorgen en steun. Ik kan onmogelijk beschrijven hoe gelukkig ik ben dat ik jullie zoon/schoonzoon ben. **Broere**, ik ben zeer trots op jou en bewonder de manier waarop je met bepaalde dingen omgaat. **Els, Bart, Vic, Joris en Lien**, ik ben zeer blij dat wij familie worden. **Karin**, lieveke, dank voor jouw luisterend oor en oppeppende woorden als ik het eventjes moeilijk had. Ik hou van de manier waarop jij kan genieten van eenvoudige dingen en hoe je zorgeloos door het leven gaat. Ik kijk echt uit naar alle mooie momenten die ons nog staan te wachten.

Dit werk kwam tot stand door de financiële steun van het Instituut voor de aanmoediging van Innovatie door wetenschap en technologie in Vlaanderen (IWT, beurs nummer 23363). De volgende bedrijven ondersteunen de ‘Clinical Epilepsy Grant’ in het Universitair Ziekenhuis Gent: **Cyberonics, UCB Pharma, Glaxo Smith Kline, Sanofi-Synthelabo, Medtronic, Novartis en Pfizer**.

Bedankt !!!